Platelet JAM-A in vascular inflammation and remodeling

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To my family

Publication

This thesis is based on the following published paper:

Hyperreactivity of Junctional Adhesion Molecule A-Deficient Platelets Accelerates Atherosclerosis in Hyperlipidemic Mice.

Ela Karshovska*, Zhen Zhao*, Xavier Blanchet, Martin M. N. Schmitt, Kiril Bidzhekov, Oliver Soehnlein, Philipp von Hundelshausen, Nadine J Mattheij, Judith M Cosemans, Remco T Megens, Thomas Koeppel, Andreas Schober, Tilman M Hackeng, Christian Weber and Rory R Koenen. Circ Res. 2015 Feb 13;116(4):587-99. PMID:25472975.

***Equal contribution**

Table of Contents

Abbreviations

ADP	Adenosine diphosphate
$Apoe^{-/-}$	Apolipoprotein e deficiency
BSA	Bovine serum albumin
CCL5	Chemokine (C-C motif) ligand 5
CD3	Cluster of differentiation 3
CD31	PECAM-1, Platelet endothelial cell adhesion molecule 1
CHO	Chinese hamster ovary
CSK	c-src tyrosine kinase
DAG	Diacylglycerol
DAPI	4'.6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESAM	Endothelial cell-selective adhsion molecule
EVG	Elastica van Gieson
FACS	Fluorescence-activated cell sorting
PF4	Platelet factor 4
FITC	Fluorescein isothiocyanate
GDP	Guanosine diphosphate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GP	Glycoprotein
GTP	Guanosine triphosphate
HBSS	Hank's Balanced Salt Solution
HFD	high-fat diet
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhsion molecule-1
IF	Immunofluorescence

IFN-γ	Interferon gamma
IL	Interleukin
IP3	Inositol 1,4,5-trisphosphate
JAM-A	Junctional adhesion molecular-A
JAM-A$^{-/-}$	Junctional adhesion molecular-A deficiency
LDL	Low-density lipoprotein
LFA-1	Leukocyte function-associated antigen 1
MAC-1	Macrophage-1 antigen
MAC-2	Macrophage-2 antigen
MCP-1	Monocyte chemoattractant protein 1, CCL2
MMP	Matrix metalloproteinase
MPV	Mean platelet volume
NF-κB	nuclear factor kappa B
NO	Nitric oxide
PAF	Platelet-activating factor
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PFA	Paraformaldehyde
PIP_2	Phosphatidylinositol-4,5-biphosphate
PKC	Protein kinase C
PMCs	Platelet-monocyte complexes
PPP	Platelet-poor plasma
PRP	Platelet rich plasma
PSGL-1	Platelet-selectin glycoprotein ligand-1
PTA	Peucutaneous translumenal angioplasty
PTP	Protein tyrosine phosphatases
PTX	Pertussis toxin
RANTES	Regulated on activation, normal T cell expressed and secreted, CCL5

RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
SDF-1	Stromal cell-derived factor 1
SH	Src homology
SMCs	Smooth muscle cells
SR-A	Scarvenger receptor A
Src Kinase	Tyrosine-protein kinase
TF	Tissue factor
TNF-α	Tumer necrosis factor
TPLSM	Two-photon laser scanning microscopy
trJAM-A$^{-/-}$	Platelet–specific JAM-A deficiency
VCAM-1	vascular cell adhesion molecule-1
VLA	Very late antigen
vWF	Von Willebrand factor
WBC	White blood cell

1 Introduction

1.1 Atherosclerosis

1.1.1 Pathogenesis of atherosclerosis

Atherosclerosis, a chronic inflammatory disease, causes plaque formation and luminal narrowing in large and medium-sized arteries and can lead to ischemia of heart, brain, or extremities, resulting in infarction[1-4]. The cardiovascular diseases due to atherosclerosis are the leading cause of death in developed countries, although the mortality rates have fallen in recent years[5]. The risks of atherosclerosis are considered as interactions between genetic and environmental factors, such as high levels of low density lipoprotein (LDL), increased blood pressure and diabetes. Under these risk factors, atherosclerotic plaque is characterized by endothelial cell activation, lipids accumulation, inflammatory cells recruitment, macrophage and foam cell formation, smooth muscle cells (SMCs) migration and extracellular matrix deposition in arterial wall[2-4].

The large and medium-sized arteries has three morphologically layers (Figure 1). The intima (innermost and very thin layer) is composed of loose connective tissue (extracellular connective tissue matrix, primarily proteoglycans and collagen) and covered by a monolayer of endothelial cells on the luminal side. Bounded by internal elastic lamina with intima, the media (the middle layer) is consisted of SMCs with regular arrangement and some extracellular collagen. The adventitia (outer layer) consists connective tissues with few fibroblasts, SMCs, micro vessels, lymphatic vessels and nerves[1].

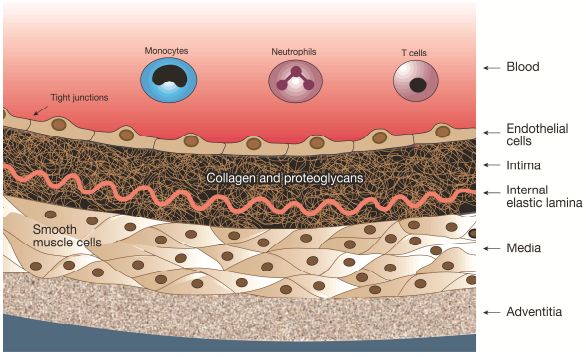


Figure1. The structure of normal large artery (Lusis AJ[1]).
This diagram shows 3 layers of a muscular artery. Normal intima is very thin (exaggerated in this figure). Middle lay maintains vessel elasticity. Adventitia consists mainly of connective tissues[1].

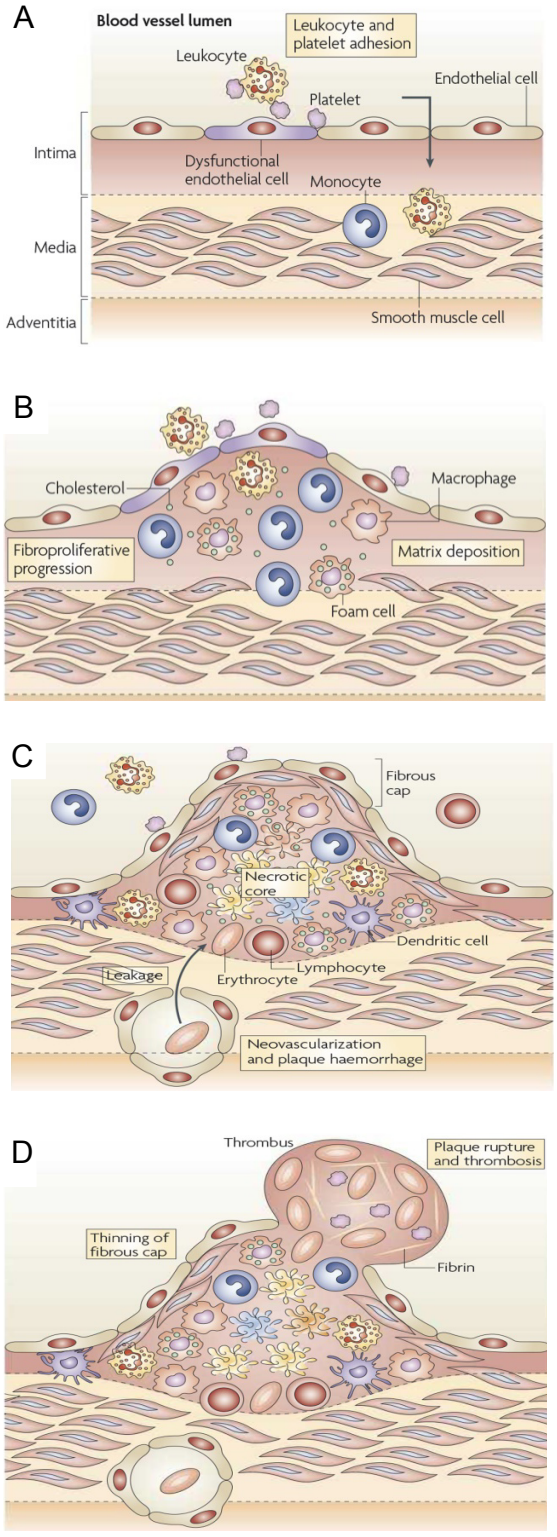
1.1.2 Various stages of atherosclerosis

Under hyperlipidemia and systemic inflammation, endothelial cells (ECs) get activated and trigger the early plaque formation (known as fatty streak formation). After activation, ECs highly express leukocyte adhesion molecules, better for leukocytes rolling, adhesion and migration into the intima[3], such as vascular cell adhesion molecule-1 (VCAM-1) upregulated before leukocyte recruitment to the areas of plaque development[6]. Moreover, ECs defect increases vascular permeability facilitating lipid components deposition, especially low-density lipoprotein (LDL). Sub-endothelial LDL is oxidized into oxidized LDL (oxLDL) and internalized by monocytes in plaque through overexpressed scavenger receptors, such as scavenger receptor A (SR-A) and CD36[7, 8]. Lipid-laden monocytes transform into macrophages and undergo a series of changes into foam cells that ultimately form fatty streaks. Furthermore, oxLDL stimulates ECs to secrete various cytokines, which drive inflammatory cells infiltration into intima[7]. The intima becomes thicker under fluid dynamics changes at atherosclerosis-prone sites, such as artery branching points. Notably, many studies have shown that platelets play important roles in this early stage as the first blood cells arriving at a scene of inflammatory endothelium[3, 9].

Following fatty streak formation, a more complex lesion evolves, which leads to clinical complications. The advanced atherosclerosis plaques are characterized by accumulation of leukocytes, lipid-laden matrix, apoptotic cells, debris and cholesterol crystals to form a necrotic core, which is surrounded by a cap[7]. This cap is called fibrous cap composed of collagen-rich extracellular matrix, SMCs, macrophages and T cells. As more extracellular matrix components (EMCs) deposition, as plaque growth is promoted by cytokines, like interleukin (IL)-1 and 6, and tumor necrosis factor (TNF)-α[10], and growth factors secreted from plaque cells. Eventually, the central core of mature plaques become necrotic, and neovascularization[11, 12]. As a result, the arterial lumen narrows (stenosis).

During plaque progression, the matrix-degrading proteases and cytokines secreted from plaque cells cause fibrous cap thinning and erosion, which may ultimately lead to plaque rupture. Once plaque rupture, various pro-thrombotic materials are exposed to blood, such as collagen, plaque debris and tissue factor, which initiate coagulation cascade and

thrombus formation, resulting in myocardial infarction, stroke or acute limb ischemia[2, 3] (Figure 2). **Figure 2: Atherosclerotic stages (Weber et. al[2]).**



A. The early stage

Endothelial cell activation in systemic inflammation leads to early platelet and leukocyte adhesion and increased endothelial permeability.

B. The intermediate stage

Monocytes i into intima and then internalize sub-intima accumulated lipids, transforming into macrophages or foam cells, which ultimately form fatty streaks. Continued mononuclear-cell influx, matrix components deposition and SMCs emigration promote fibrous proliferation.

C. The advanced stage

Apoptosis of macrophages and other plaque cells leads to necrotic core formation, paralleled with a fibrous cap covering on the luminal side that consists of matrix and SMCs layer. Neovascularization can occur within plaque on the adventitial side, and leakage of these fragile vessels can lead to intra-plaque hemorrhage.

D. The complication

Thinning and erosion of the fibrous cap in unstable plaques, for example, owing to matrix degradation by proteases, ultimately results in plaque rupture, with debris release, coagulation system activation and thrombosis in artery. This results in arterial occlusion and myocardial infarction or stroke.

1.2 Neointimal hyperplasia following vascular injury

Compared to chronic and lipid-induced atherosclerosis, acute mechanical injury also leads to arterial narrowing. The vascular stenosis, defined as narrowing more than 50% of luminal diameter, is a major clinical pathogen of various cardiovascular events associated with arterial ischemia. Current therapeutic approaches to restore blood flow mainly include percutaneous transluminal angioplasty (PTA), stenting and bypass surgery, which mechanically injury the vessel wall. Although these treatments effectively relieve stenosis after operation, the recurrence of stenosis (restenosis) becomes a main clinical challenge. The high incidence rate is 25-50% in patients after balloon angioplasty with the majority undergoing revascularization within 6 months[13, 14]. To limit the high rate of restenosis after balloon angioplasty, placing a stent into the stenosis was introduced. Coronary stenting significantly improved this outcome, as a 10% reduction of restenosis rate compared to angioplasty alone[15]. Currently the drug eluting stents are widely implemented, which have substantially reduced the restenosis rate. Despite considerable development of therapy progress, the mechanisms of restenosis (neointimal hyperplasia) remains incompletely understood.

One hypothesis is used to describe some mechanisms that lead to restenosis. In this hypothesis, vascular injury causes endothelial denudation, exposure of extracellular matrix, platelet adhesion, thrombus formation, leukocytes infiltration[16], and SMCs proliferation, migration and secretion of extracellular matrix[17]. Highly proliferative SMCs migrate into intima, undergoing phenotypic modification from contractile to synthetic[15], and this is known as the main character of neointimal hyperplasia. It is drived by growth factors, such as transforming growth factor-β and platelet-derived growth factor (PDGF)[18], and cell cycle genes, including upregulated proliferating cell nuclear antigen (PCNA) in SMCs to promote cell growth[19], and highly expressed matrix metalloproteinases (MMPs) to facilitate SMCs migration from media to intima[20]. Despite this, the inflammatory concept is more and more accepted now.

Endothelium plays pivotal roles in suppression of neointimal hyperplasia by inhibition of inflammation, thrombus formation and SMCs proliferation and migration[21]. After injury, the wound healing process requires endothelial repair. Re-endothelialization was classically considered as the migration and proliferation of adjacent endothelial cells to cover injured vessel wall. However, mature resident vascular endothelial cells have very limited proliferation capacity[22]. Thus, the whole endothelial repair might also dependent on circulating bone marrow-derived cells, termed endothelial outgrowth cell (EOCs), which can be recruited to the sites of damaged endothelium and differentiate into ECs[23]. EOCs can be derived from circulating endothelial progenitor cells, hematopoietic stem cells and myeloid cells[24]. As an endogenous repair mechanism, they also play an important role to replace damaged endothelium[25]. Although re-endothelialization protects from neointimal formation, dysfunctional endothelial cells are also facilitating leukocytes recruitment under inflammatory conditions[26].

Platelets and leukocytes release cytokines, chemokines, vasoactive agents, and growth factors, which promote inflammatory response to vessel injury[27]. During neointimal formation, activated platelets secrete cytokines and growth factors, which promote inflammatory recruitment and SMCs accumulation[26]. Inflammation accompanies with neointimal formation after acute vascular injury and thrombus deposition during the chronic healing process[28], which can be characterized by three stages: early thrombotic phase, intermediate recruitment phase, and late neointimal proliferation phase[29].

In early phase, angioplasty and stenting cause endothelial denudation and dysfunction. Consequently, sub-intima exposure activates platelet aggregation, followed by fibrin deposition and thrombus formation[30]. In turn, thrombus provokes inflammatory process. In intermediate phase, lots of leukocytes are recruited to damaged vessel wall by adhesion molecules and chemokines[15]. Firm adhesion and trans-platelet migration of leukocytes are mediated by integrins[31]. For example, integrin molecule MAC-1 (CD11b/CD18) mediates leukocytes firm adhesion to endothelial counter ligands (eg, intercellular adhesion molecule-1 [ICAM-1]), to endothelial associated extracellular matrix proteins (eg, fibrinogen), or to glycosaminoglycans[28, 32], and also to platelets via GPIbα[33]. Chemokines recruit leukocytes to vessel wall, which may ultimately lead to

neointimal formation. For example, monocyte chemoattractant protein 1 (MCP-1, CCL2) as an arrest chemokine is linked to adherent platelets, thus locally concentrated on the denudated endothelium, which can recruit monocytes[34]. Interestingly, CCL2 is upregulated in ECs and SMCs after stenting[35] and the levels of CCL2 are persistently elevated in patients with restenosis[36]. In late phase, neointima is characterized by new extracellular matrix accumulation in intima, mainly collagen.

Platelet plays a pivotal role in neointimal formation. Activated platelets release cytokines that activate endothelial proliferation, recruit leukocyte and promote SMCs migration from the media to the intima, thus it endows complicated functions. Recently, it was further characterized in wire-induced carotid injury of apoe$^{-/-}$ mice. After carotid injury, CCL5 (RANTES) released from platelets and deposited on ECs in neointimal lesions facilitates monocyte migration. Treatment with selective CCR1 and CCR5 antagonist of Met-CCL5 clearly reduces macrophage infiltration and neointimal formation, consistent with inhibition of CCL5 expression in vivo. Furthermore, it has been found that CCR5 but not CCR1 appears to be responsible for CCL5-mediated macrophage infiltration and neointimal formation[37, 38].

Junctional adhesion molecule-A (JAM-A) is widely distributed and expressed on platelets, erythrocytes, lymphocytes, neutrophils, and monocytes, which has recently been implicated in leukocyte recruitment on early atherosclerotic endothelium and promotion for neointimal formation[39]. The globally genetic deletion of JAM-A in apolipoprotein e–deficient (aope$^{-/-}$) mice significantly reduced neointimal hyperplasia, which was associated with a significant decrease of neointimal macrophages. Now we found the cell-specific roles of JAM-A in atherosclerosis[40], indicating that JAM-A functions in different cell types might be potentially various in neointimal formation.

1.3 Leukocyte migration into the vessel wall

During whole stages of atherosclerosis (Figure 3), leukocytes migration into vessel wall is the main driving force of plaque growth in combination with hyperlipidemia. Lots of leukocytes are found in plaques, particularly monocytes (macrophages) and T lymphocytes[41-43]. During attraction of leukocytes, many families of chemoattractant cytokines (chemokines) are defined and capable to recruit leukocytes into arterial intima, for example, CCL2 overexpressed in plaque can recruit monocytes to the lesion site[44, 45]. Targeting molecules involving in leukocyte recruitment would be a novel and beneficial therapy for atherosclerosis[46].

Leukocyte adhesion and transmigration through vessel wall consists many steps, including capture or tethering, slow rolling, arrest, intraluminal crawling and transmigration. The first adhesion step is tethering to endothelial cells, which is mediated by leukocyte-, platelet-, and endothelial- selectin (CD62-L, -P, and -E) binding to their ligands, platelet-selectin glycoprotein ligand-1 (PSGL-1) and other glycosylated ligands. Selectins bind to their ligands in a calcium-dependent manner. Leukocyte PSGL-1 interacting with endothelial E- and P- selection and the same leukocyte L-selectin binding to endothelial PSGL-1 enable leukocyte captured and tethering to endothelium. Importantly, activated platelets also express P-selectin assisting leukocyte tethering[47-49]. The bonds of selectins with their ligands can be formed or broken dependent on the conditions of blood flow. For example, shear stress determinates L-selectin and P-selectin interactions with their ligands to support leukocyte adhesion; the tethering cells can detach from endothelium when blood flow is stopped[50, 51], indicating inhibition of low shear stress to the interactions. Rapid ligand interaction and dissociation rate of selections enable leukocytes tethering or rolling along endothelium under blood shear flow[52].

During cell tethering, the leukocytes are rolling along endothelium to search for suitable adhesion sites, marked by chemokine gradients and characterized by high cell adhesion molecule (CAM) expression. Beside E- and P-selectin assisting leukocyte rolling, integrins also mediate leukocyte rolling and further firm adhesion, such as $\alpha_4\beta_7$-integrin–mucosal vascular addressin cell-adhesion molecule (MADCAM)-1 interaction, very late

antigen (VLA)-4–VCAM-1 interaction[53] and lymphocyte function-associated antigen (LFA)-1–intercellular adhesion molecule (ICAM)-1 interaction[54]. Meanwhile cytokines activate inflammatory signaling pathways for subsequent steps in leukocyte adhesion cascade like adhesion stabilization, crawling and transmigration.

After rolling step, integrin interaction with adhesion molecular mediates leukocytes arrest and firm adhesion on endothelium by increasing integrin affinity. For example, LFA-1 and VLA-4 change their structures from a bent low affinity to an opened intermediate- or high affinity conformation, enabling them to bind their ligands ICAM-1 and VCAM-1 respectively[55, 56]. Interestingly, activated endothelial cells and platelets can secrete lots of chemokines, which are presented on endothelium and interact with G-protein coupled receptors (GPCRs) to induce integrin conformation changing[57-59]. For example, CXC-chemokine ligand 4 (CXCL4), CXCL5 and CC-chemokine ligand 5 (CCL5; previously known as RANTES) secreted from platelets are deposited on the endothelium to interact with GPCRs on passing leukocytes inducing integrin high affinity states to facilitate leukocyte arrest[60, 61]. Chemokines trigger GPCRs-related signaling of integrin conformation changing, which is referred as inside-out signaling, in contrast to outside-in signaling, which is referred as ligand binding to activated integrin for cell adhesion strengthening and motility.

Subsequent to firm adhesion of leukocytes on the endothelium, leukocytes crawl into the endothelial junctions or endothelial cell bodys. Leukocytes are stimulated by chemokines in intima to migrate through endothelial monolayer. This complicated step involves many molecules, such as CD9, CD151, CD81, cytoplasmic molecules like ERM (ezrin, radixin, moesin) proteins, and cytoskeletal components like vinculin, α-actin and talin-1. ICAM-1 binding to integrin ligand MAC-1 can active the signaling cascade, through intracellular Ca^{2+}, p38 mitogen-activated protein kinase (MAPK) and RAS homologue (RHO) GTPase, which can lead contraction of endothelial cells and opening of inter-endothelial junctions for leukocyte migration[62-65].

The last step of leukocyte adhesion cascade is leukocyte trans-endothelial migration, which takes less time (<2-5 minutes) than migration through the basement membrane (>5-15 minutes)[66]. During migration, the cell-cell junctions are opened transiently to

allow leukocyte crossing[67]. Actually, two routes of leukocyte diapedesis across endothelium are found: one paracellular route through junctions of adjacent ECs and one transcellular route through EC body directly[68]. However, paracellular pathway might be the dominant route, which is mediated by homophilic and heterophilic interactions between cell adhesion molecules and integrins. During paracellular migration, the junctional cell adhesion molecules can be re-localized away from cell-cell junction to the luminal site to weaken junctional barrier[69] and facilitate firm adhesion of leukocytes to ECs[67]. ECs form "migratory cups" by integrins, such as ICAM-1 and VCAM-1[70], which are re-located and concentrated on endothelial surface. Leukocytes adhesion and binding to ICAM-1 and VCAM-1 cause endothelial actin stress-fiber formation and increased permeability controlled by GTPases RhoA, Rho1 and Rap1[71]. This progression involves a number of cell adhesion molecules interactions. Importantly, Junctional adhesion molecule (JAM)-A, JAM-B, JAM-C and endothelial cell-selective adhesion molecule (ESAM) also promote trans-endothelial migration via homophilic and heterophilic interactions with integrins[67, 72, 73]. Leukocyte migration though endothelial basement membrane across gaps between adjacent pericytes and regions of low protein deposition within extracellular matrix is facilitated by integrins and proteases, such as matrix metalloproteinase (MMPs).

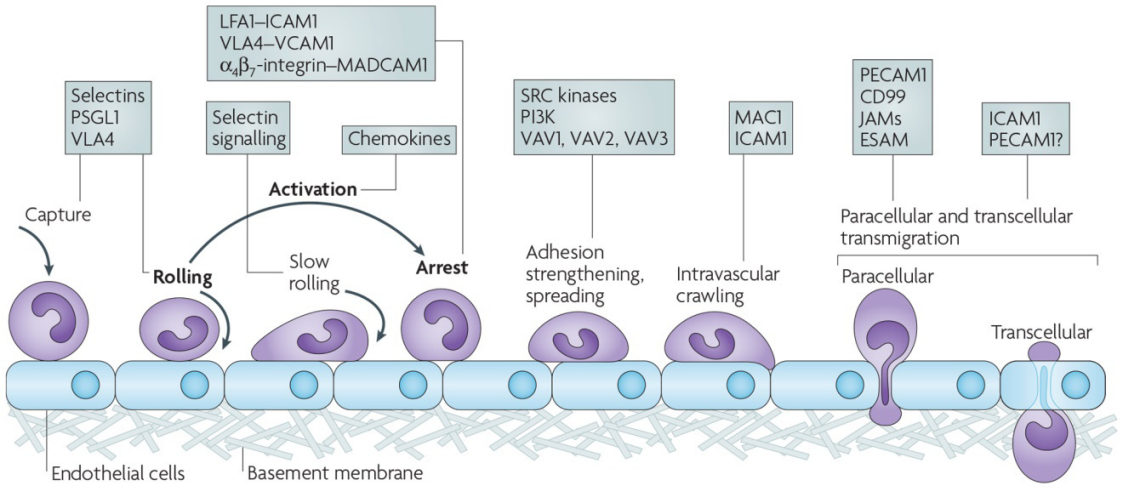


Figure 3: The leukocyte adhesion cascade (Ley K et al[66]).

The traditional three steps of leukocyte adhesion are marked in bold. Rolling is mediated by selectins. Activation is mediated by chemokines. Arrest is mediated by integrins and immunoglobulin-superfamily members. Progress of leukocyte infiltration into vessel wall is defined in many steps: capture (or tethering), slow rolling, adhesion strengthening and spreading, intravascular crawling, and paracellular or transcellular transmigration. Leukocytes are directed by chemokines in flow or by immobilized chemokines gradients to migrate across the endothelium.

Important molecules are described. ESAM, endothelial cell-selective adhesion molecule; ICAM1, intercellular adhesion molecule 1; JAM, junctional adhesion molecule; LFA1, lymphocyte function-associated antigen 1; MAC1, macrophage antigen 1; MADCAM1, mucosal vascular addressin cell-adhesion molecule 1; PSGL1, p-selectin glycoprotein ligand 1; PECAM1, platelet/endothelial-cell adhesion molecule 1; PI3K, phosphoinositide 3-kinase; VCAM1, vascular cell-adhesion molecule 1; VLA4, very late antigen 4[66].

1.4 Platelets in vascular inflammation

Platelets, also called "thrombocytes", are first characterized in 19th century as "small, curious beads or grains which accumulate with each other to irregular cluster" and nowadays are well known as anucleate cellular fragment shaped like a lens. The resting platelets at a diameter of 2-4μm are derived from megakaryocytes in bone marrow and show a lack of genomic DNA[74], but contain megakaryocyte-derived mRNA for protein synthesis[75]. Platelet was initially only believed to play a pivotal role in physiological hemostasis and pathophysiological thrombosis, protect against bleeding and promote the stable blood clots formation via the coagulation cascade. Then it is considered important for thrombus formation upon plaque rupture. Now, these concepts have been expanded that platelets also assist and modulate inflammatory reactions, immune response, and atherosclerosis progression[76-78].

The vascular inflammation is a key process of atherosclerosis, accompanied and amplified by platelet activation and consequent interactions of platelets with ECs and leukocytes. Platelet interactions with leukocytes and ECs are regarded as important factor in the development and progression of atherosclerosis and neointimal formation. In the lesion sites, platelet-derived chemokines, with upregulated adhesion molecules, promote platelet adhesion and leukocyte recruitment, which eventually migrate into vessel wall. Under inflammatory conditions, platelets may already become activated in circulation or form platelet-leukocyte complexes with augmented capacities of adhesion and migration[78]. For example, it has been found that activated platelets can trigger and accelerate atherosclerosis in hyperlipidemic apoe$^{-/-}$ mice, involving platelet surface receptors to promote recruitment of monocytes[9, 60]. The mechanisms underlying platelet-leukocyte interactions involve a number of important molecules, for instance, selectins with their ligands, and integrins binding to Ig-like cell adhesion molecules as well as to glycoprotein, importantly von willebrand factor (vWF), which can be found in plasma, extracellular matrix and activated endothelial cells. So the platelet can been considered as pivotal mediator in vascular inflammation.

Various studies show that platelets are initiators and propagators of atherosclerosis[60], by release of cytokines and interactions with other cells, promoting recruitment of various inflammatory cells to the vessel wall and fostering processes such as atherosclerosis, neointimal formation and thrombosis, but also vessel repair and regeneration after vascular injury. For example, Henn and colleagues found that platelets expressed CD154 within seconds after activation in vitro and in the process of thrombus formation in vivo. Interestingly, CD154 on platelets was found to stimulate ECs to secrete chemokines and express adhesion molecules, thereby generating signals for the recruitment and extravasation of leukocytes at atherosclerotic predilection sites. Thus, platelet can directly initiate an inflammatory immune response. In addition, IL-1β secreted by platelets can induce expression of CCL2 and ICAM-1 in ECs[79, 80], indicating the alterations of chemotactic and adhesive properties of ECs due to platelet activation. In carotid arteries of apoe$^{-/-}$ mice, platelets were shown to interact with endothelium of atherosclerosis-prone sites in GPIbα- and integrin αIIbβ3-dependent manner. These interactions appeared to precede expression of adhesion molecules and subsequent extravasation of monocytes, ultimately leading to plaque development[9]. Transient platelet-endothelium interactions are sufficient for the deposition of the chemokines CCL5 and CXCL4[81], which further propagates plaque development[60, 82]. Under transient interactions mediated by P-selectin, adherent platelets form an adhesive substrate for inflammatory cells, such as neutrophils, and subsequently secrete platelet-activating factor (PAF) to stimulate neutrophils adhesion and spreading on endothelium[83]. P-selectin on platelets also plays an important role in cell interactions and promotes the fatty streaks formation at atherosclerotic early stage[84]. Activated platelets activate endothelium, inhibit endothelial regeneration and promote foam cell development[85], playing an important role in the vascular inflammation.

1.5 Platelet interaction with endothelial cells and leukocytes

The transient and stable interactions of platelets with ECs depend on different platelet receptors, which can result in the development and progression of atherosclerosis[78]. However, health, non-activated endothelium can prevents platelets adhesion by its antithrombotic properties, involving releasing platelet activation-inhibiting substances

such as NO, prostacyclin and cyclooxygenase-2[86]. After endothelial cell activation, it can change phenotype from anti-thrombotic properties to pro-thrombotic properties, characterized by releasing of platelet-binding and stimulating agents, such as ADP, vWF, tissue factor (TF) and adhesion molecules[87]. Firstly, transient rolling is mediated by platelets GPIb with P-selectin, which is a cell adhesion molecule expressed by platelets, ECs and macrophages[88]. P-selectin is stored in granules called Weibel-Palade bodies of un-activated ECs or in α-granules of un-activated platelets, which can be rapidly transported to cell surface after activation, allowing leukocytes and platelets interaction quickly with ECs during inflammation. Another primary ligand of P-selectin is P-selectin glycoprotein ligand-1(PSGL-1) expressed on almost all leukocytes, platelets and endothelial cells[89]. Beside the traditional way of platelet rolling mediated by P-selectin-PSGL-1, transmembrane GP EMMPRIN (CD147) on activated platelets with endothelial counter-receptors GPVI[90] and E-selectin[91], can also mediate platelets rolling. Subsequently, stable adhesion of platelets to inflamed endothelium mediated by additional molecules, such as GPIb-IX-V with vWF and GPVI with collagen interactions respectively, results in platelet activation, characterized by integrins αIIbβ3 (GPIIbIIIa, fibrinogen receptor) and α2β1 (collagen receptor) activation[92]. The activated integrins are essential and required for stable adhesion of platelets to extracellular matrix and ECs. In addition, the integrins on platelets can directly bind to collagen, vWF and endothelial adhesion molecules, or indirectly bind via additional bridging molecules, such as fibrinogen, fibronectin or vWF, to endothelial activated ICAM-1, αvβ3 integrin, and GPIb, respectively[93, 94]. Meanwhile, novel mechanisms have shown that membrane-bound chemokine CX3CL1 expressed on inflamed endothelial cells can stabilize platelet-endothelium interactions[95]. Except heterophilic interactions, platelets can also interact with ECs by molecular homophilic interactions, such as JAM-A-JAM-A interaction[96].

Platelet adhesion induces endothelium pro-inflammatory phenotype, marked by platelet spreading and activation, upregulated expression of adhesion molecules, such as P-selectin, CD40L, TNFSF14 and secretion of pro-inflammatory substances, such as IL-1β and CXCL4[78, 97]. IL-1β can in turn induce ECs to secrete CCL2, GM-CSF, IL-6 and to upregulate expression of ICAM-1 and αvβ3 integrin, involving activated NF-κB

pathway[79, 98]. Additionally, CD40L (CD154), which is stored in significant amounts in platelets and released within seconds by αIIbβ3 activation, can bind to endothelial CD40, including increased secretion of endothelial CXCL8 and CCL2, expression of adhesion molecules, urokinase type plasminogen activator (uPAR) and matrix metalloproteinase (MMP)-2 and -9, and production of reactive oxygen species (ROS)[99-101].

Except platelet-endothelium interaction, activated platelets also show robust interactions with leukocytes, characterized by increase of circulating platelet-leukocyte complexes. In particular, increased platelet-monocyte complexes (PMCs) have been observed in clinical conditions, such as peripheral vascular disease, hypertension[102], acute or stable coronary syndrome[103-105], stroke[106], or diabetes[107]. Conversely, high dietary intake of omega-3 fatty acids reduces activated platelets and PMC levels, associated with reduction of cardiovascular events[108]. The presence of PMCs is not only a sensitive marker for platelet activation and cardiovascular diseases but is also regarded more and more as a cardiovascular risk factor[106, 109].

Platelets binding to monocytes shows more and initial faster, compared with neutrophils, so the researches focusing on PMCs are more intensive. P-selectin expressed on surface of activated platelets mediates the binding to monocytes via PSGL-1, which determinate the extent of PMCs formation[110]. The binding of platelets to monocytes via P-selection-PSGL-1 induces L-selectin shedding from the monocyte surface and upregulates expression and activity of integrins α4β1 and αMβ2 (Mac-1)[111], which in turn stabilizes the adhesion via MAC-1 to the GPIbα. Similarly, involvement of CD40-CD40L increases the expression of β1- and β2-integrin for platelet-monocyte adhesion and recruitment into the injured vessel wall[112]. In addition, activated platelets via P-selection-PSGL-1 or GPIbα-MAC-1 interactions can induce an inflammatory cascade in monocytes, such as upregulated expression and secretion of chemokines via NF-κB activity[113-115]. Furthermore, the chemokines from activated platelets can be deposited on monocytes in PMCs, which induce monocytes activation, adhesion molecules expression and cytokines secretion, such as TNF-α and CXCL8[116, 117]. Importantly, PMCs have been upregulated

integrin expression and activity compared with platelet-free monocytes and increased monocyte adhesion and transmigration capacity[118].

1.6 Platelet chemokines

Chemokines are a family of small chemotactic cytokines, with a molecular weight ranging from 8-10 kDa, containing 60-100 amino acids. There are approximately 50 different chemokines, which are classified into four subclasses, CXC-, CC-, C- and CX_3C-chemokines, based on the relative position of the first consensus cysteins on N-terminal part of proteins. Chemokines regulate cell functions, including immune survival, development, hematopoiesis, wound healing, inflammation, viral infections or metastases[119]. Especially, the trafficking and extravasation of leukocytes are governed by chemokines during immune surveillance and inflammation.

Chemokine receptors are large proteins binding different chemokines and chemokines themselves are able to bind several different receptors. All chemokine receptors are specific cell surface G-protein-coupled receptors (GPCRs), consisting of about 350 amino acid residues and sharing common structural features: an extracellular amino-terminus (N-terminus), a polypeptide which loops across the membrane seven times to form three intracellular and three extracellular loops, a disulfide bond linking cysteine residue in the first and second extracellular loops and last an intracellular carboxyl-terminus (C-terminus)[120]. Moreover, most chemokines also display a high affinity to a class of polysaccharide called glycosaminoglycans (GAGs). GAGs are present on all cell surfaces and in intracellular matrix, which can covalently attach to core proteins[121]. This bound facilitates chemokines immobilization and gradients formation[122]. When chemokine binds to its receptor, conformational changes are induced and result in receptor activation and signal transduction.

Platelet-derived chemokines are synthesized in megakaryocytic precursor cells and stored in α-granules of mature platelets, regulating inflammatory progress, atherosclerosis, neointimal formation and angiogenesis. PF4 (CXCL4) was the first chemokine

discovered in releasates from platelets[123], involving in proliferation, apoptosis and differentiation of immune cells[124]. CXCL4 can bind to a 200 kDa chondroitin sulfate proteoglycan on the surface of human neutrophils,[125] and has also been shown to bind to CXCR3B, a splice variant of CXCR3, but not to CXCR3A, which is a receptor expressed on ECs and T cells, not on monocytes or neutrophils[126]. However, the chemotactic activity of human T cells towards to CXCL4 is mediated by both CXCR3 variants in a PTX-sensitive manner[127]. Human neutrophil adhesion in response to CXCL4 is blocked after treatment with a Src kinase inhibitor or other inhibitors for Syk, RAS and JNK, whereas PF-4-mediated exocytosis requires the additional activation of p38 MAP kinase and phosphatidylinositol 3-kinase[128]. These findings suggest that CXCL4 can trigger more than one signaling pathway.

In atherosclerosis, CXCL4 was found in early and late lesions of carotid artery, correlating with clinical outcome[129]. CXCL4 deficiency in apoe$^{-/-}$ mice or transplantation of CXCL4 deficient bone marrow into apoe$^{-/-}$ mice decreased atherosclerotic lesions formation with reduced macrophage infiltration[82]. However, since highly purified CXCL4 lacks chemotactic activity for neutrophils, monocytes and T cells[130], the mechanism of CXCL4 influence on atherosclerosis needs more investigation. Additionally, CXCL4 have been described to induce cells differentiation, such as monocyte differentiation to macrophages[131] or specialized antigen-presenting cells[132], apart from its potential role in conjunction with CCL5 in monocyte recruitment to vessel wall[133]. So CXCL4 may increase atherogenesis by promoting differentiation of monocytes into macrophages and foam cells. Furthermore, CXCL4 induces higher phagocytic capacity as compared to GM-CSF-induced macrophages[134]. Some experiments found that CXCL4 can bind to the oxLDL particles, resulting in increased uptake by macrophages and foam cell formation[135], which would be expected to promote foam cell formation in atherosclerotic lesion. Additionally, CXCL4 exerts effects on inhibition of cell proliferation and angiogenesis in ECs[136]. This inhibition was also shown that CXCL4 inhibits proliferation and cytokine release from activate T cells in vitro[137].

CCL5 (RANTES) is a chemokine with a molecular mass of 7.8 kDa and secreted by various cell types such as ECs, SMCs, macrophages, activated T cells and platelets. Binding to CCR1, CCR3, CCR4 and CCR5 receptors and inducing transient calcium influx, CCL5 plays an important role in monocyte and T lymphocyte recruitment in inflammation and atherosclerosis[138, 139]. The signal transduction induced by CCL5 in T cells results in focal adhesion and subsequent cell activation via a molecular complex containing focal adhesion kinase, the tyrosine kinase zeta-associated protein (ZAP) 70 and paxillin[140]. Additionally, after exposure to CCL5, the intracellular intergrin-domain of monocytes and lymphocytes are phosphorylated for the adhesion[141]. In neointimal lesions after wire-induced carotid injury of apoe$^{-/-}$ mice, CCL5 has been primarily detected on ECs and was possibly deposited from activated platelets and neointimal SMCs. Most notably, CCR5 has been shown as a potential therapeutic target of anti-inflammation, for example, CCR5 antagonist maraviroc (UK-427857) is admitted as an HIV-inhibitor and was shown to reduce atherosclerosis in the murine system without affecting lipid levels[142].

1.7 Junctional adhesion molecules

1.7.1 Junctional adhesion molecules in inflammation

Junctional Adhesion Molecules (JAMs) are transmembrane proteins belonged to immunoglobulin superfamily. JAM-A (CD321, JAM-1 or F11 receptor) initially called F11 receptor was recognized as a receptor for platelet activating antibody F11[143]. Other family members are JAM-B, JAM-C, JAM-4, ESAM and JAM-like (JAM-L) proteins. They are expressed in tight junctions of endothelial and epithelial cells and also in circulating cells that do not form junctions, such as leukocytes and platelets. These proteins control vascular permeability by regulating barrier function of tight junctions and facilitate leukocyte transmigration across endothelium through homophilic, heterophilic and lateral interactions. Their expression, location and function are regulated by inflammatory cytokines and growth factors, and they also translate extracellular adhesive events into functional responses. Antibody-blocking studies and studies using

genetically modified mice have implicated JAMs functions in regulation of leukocyte recruitment to sites of inflammation and ischemia-reperfusion injury, and in vascular remodeling, such as growth-factor-mediated angiogenesis, atherosclerosis and neointimal formation. JAMs may regulate leukocyte recruitment by redistributing to luminal surface under inflammatory conditions and may promote leukocyte movement through intercellular junctions. However, the signaling cascades through JAMs remain incompletely clear. Furthermore, their functions are considerably various in different tissues under cell-specific and context-dependent conditions among different members of the JAMs (Figure 4)[73].

JAMs have an extracellular domain with two immunoglobulin-like domains (membrane-distal V-type and membrane-proximal C2-type), a single transmembrane segment and a short cytoplasmic tail with a phosphorylation site for protein kinase C (PKC) and a PDZ-domain-binding motif linking to tight junction-associated scaffold proteins such as zonula occludens 1(ZO-1), afadin 6 (AF-6), ASIP/Par3, cingulin.

Increasing evidence points a role of JAMs in vascular inflammation. An up-regulation of JAMs expression on atherosclerotic endothelium and early lesions has indicated a role of JAMs in plaque formation, such as increased expression of JAM-A and JAM-C at the protein and transcript level induced by oxidized lipoproteins (rather than cytokines) under hyperlipidaemic conditions in apoe-deficient mice[144, 145]. JAM-C on atherosclerotic endothelium can mediate MAC1-dependent transmigration of leukocytes[98], and a function of JAM-A in early atherogenesis was inferred by soluble JAM-A inhibiting leukocytes accumulation[138]. Furthermore, up-regulation of JAM-A expression in unstable human plaques concomitant with increased macrophage infiltration further implies its role at later stages of lesion development and progression[146, 147], indicating its involvement in the rupture of atherosclerotic plaque.

Apart from directly contributing to leukocytes recruitment, JAMs expressed on platelets have additional mechanisms that regulate atherosclerosis progression. Platelet JAM-C may promote atherothrombosis through the engagement of MAC-1 to mediate leukocyte-

platelet interactions or to promote the recruitment of such complexes[148]. JAM-A on the surface of platelet was found to participate in platelet adhesion to endothelium under inflammatory conditions[96]. Transient interactions of platelets with early atherosclerotic endothelium can lead to endothelial deposition of platelet chemokines, such as CCL5, which trigger other platelet-dependent mechanisms and leukocytes recruitment to aggravate atherosclerosis[60, 81]. Moreover, PMPs shed from activated platelets mediate CCL5 deposition by transient tethers on activated endothelium, also involving JAM-A signals[149]. Indeed, CCL5 deposition by JAM-A deficient platelets on endothelium is impaired[39]. Thus, platelets use JAM-A engagement during interactions with ECs. In addition, JAM-A has also been identified on SMCs in atherosclerotic lesions, but its contribution remains unclear[145]. Most notably, how JAM-A regulates platelet activity in atherosclerosis attracts us to investigate.

JAM-B expression is restricted to ECs and localizes mainly in the junctions of high endothelial venules and of heart, and not detected in leukocytes. Additionally, JAM-B is more expressed in chronic inflammatory tissues in asthma, bronchitis, interstitial nephritis, autoimmune hepatitis and alcoholic cirrhosis[150]. JAM-B heterophilically interacts with integrin VLA-4 and JAM-C, but JAM-B–VLA-4 interaction depends on JAM-C. For example, T cells lacking functional JAM-C did not show any JAM-B–VLA-4 interaction[151, 152]. Furthermore, JAM-B and -C are involved in leukocyte extravasation to sites of inflammation, determined by the evidence that JAM-B and -C play a role in leukocyte extravasation to the inflammation of skin. JAM-B and -C have overlapping, but distinct functions in regulating leukocyte transmigration. JAM-B may predominately govern neutrophil transmigration, whereas JAM-C may govern edema formation, however, further studies are required to investigate molecular mechanisms of JAM-B and -C mediated leukocyte migration in vascular inflammation[153].

JAM-C is expressed in ECs and lymphocytes including monocytes, natural killer cells, dendritic cells, B cells and T cells. Through hemophilic and heterophilic interactions with JAM-B and MAC-1, JAM-C plays an important role in vascular inflammation through regulating leukocyte transmigration. Woodfin et al. found that JAM-C rather regulates

polarized leukocyte transmigration in a sense that it blocks leukocyte reverse endothelial transmigration[154]. Furthermore, increased expression of JAM-C was found in atherosclerotic lesions of apoe$^{-/-}$ mice, together with a re-localization away from endothelial cell junctions to the apical site[144]. Blocking JAM-C decreased neointimal hyperplasia after wire injury of carotid arteries[155].

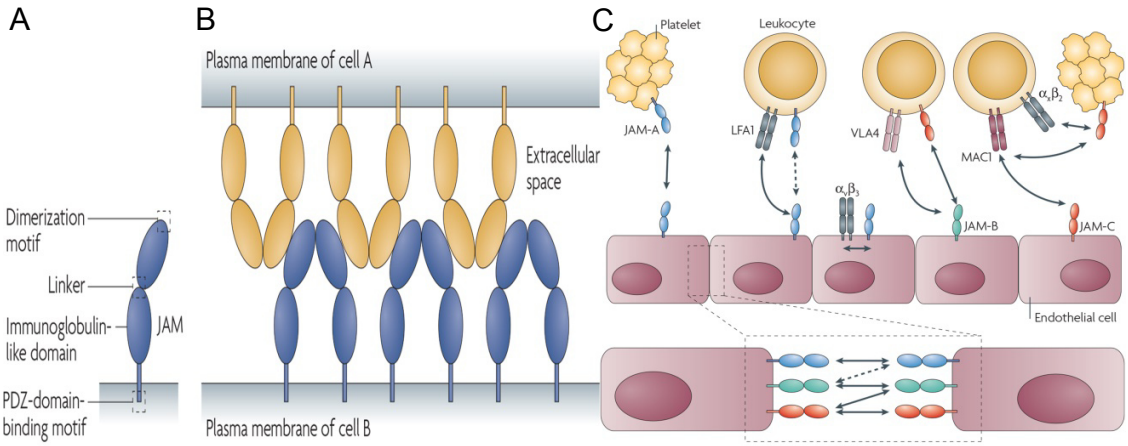


Figure 4: Structural features, homophilic adhesion and extracellular ligands of JAMs. (Weber et al.[73])

A. JAMs structure contains two immunoglobulin-like domains in extracellular portion, a single transmembrane segment and a short cytoplasmic tail with a PDZ domain-binding motif (Phe-Leu-Val). The short linker sequence (Val-Leu-Val) connects two immunoglobulin domains to impose a bent conformation. The dimerization motif (Arg-Val/Leu/Ile-Glu) in membrane-distal domain is essential for homodimer formation. **B.** Homophilic interactions of JAM-A in cis (via dimerization motif in the membrane-distal immunoglobulin domain) in a shape of an inverted 'U'. These cis-homodimers bind in trans to JAM-A homodimers of an adjacent cell surface at intercellular junctions. **C.** 1) Homophilic and heterophilic interactions of JAM-A, JAM-B and JAM-C regulate endothelial permeability. 2) Homophilic interaction of JAM-A mediates platelet adhesion to endothelium. Heterophilic interactions of JAM-C–integrins support platelet adhesion to leukocytes. 3) Crosstalk of JAMs and integrins supports leukocytes binding to endothelium, such as the interactions of integrin lymphocyte function-associated antigen 1 (LFA1) with JAM-A, very late antigen 4 (VLA4) with JAM-B, and JAM-C with MAC1.

1.7.2 Junctional Adhesion Molecule A

Murine and human JAM-A was first characterized in 1998 and 1999 respectively[156, 157]. JAM-A sharing about 70% amino acid identity is highly conserved among mammals[156]. In extracellular domain, two Ig-like domains are connected by a linker of 3 amino (val-leu-val) to form a bent 125^0 conformation, which is stabilized by hydrogen bonding. The membrane-distal V-type domain contains a dimerization motif, major for homodimer formation.

JAM-A is mostly abundant in tight junctions of endothelial and epithelial cells in various organs including liver, kidney, pancreas, heart, brain, lymph nodes, intestine, lung, placenta and vascular tissue[156]. Furthermore it is expressed in lymphoid and myeloid origin[157]. Inflammatory cytokines regulate JAM-A junctional, such as TNF-α and IFN-γ treatment of endothelial cells leading JAM-A re-localization from intercellular junctions to luminal surface, although expression levels remains unchanged[69, 156]. In vivo, JAM-A expression is upregulated on atherosclerotic endothelium and in unstable atherosclerotic plaques[146, 158]. Similarly, JAM-A expression is increased on hepatic venular endothelium after ischemia-reperfusion injury[159]. JAM-A re-localization might be involved in atherosclerosis[40]. In another study on brain endothelial cells, CCL2 induced JAM-A re-distribution from inter-endothelial cell area to apical surface, associated with internalization via micropinocytosis during para-cellular route opening. Internalization of JAM-A within a short time period (<10 min) and redistribution of most internalized JAM-A to the brain endothelial cell apical membrane involve Rho family kinase as well as actin reorganization[160].

JAM-A displays different patterns of homo- and heterophilic adhesions. The interaction and function of JAM-A with its partners play an important role in regulation of endothelial permeability and are therefore a pivotal component of inflammatory reactions. An over-expression of JAM-A increases endothelium barrier function by interaction in a homophilic manner with neighboring molecules (in cis) and in dimeric state with other dimers from adjacent cells (in trans), forming a tight, zipper-like structure. It also

interacts with β2 integrin leukocyte function-associated antigen 1 (LFA-1; integrin αLβ2; CD11a; CD18), which is associated with trans-endothelial cell migration and leukocytes recruitment, such as monocytes and neutrophils (figure 4)[161]. A typical feature of JAM-A in endothelial-leukocyte interactions is a contribution to specific ring-like structure that forms on ECs surrounding a transmigrating leukocyte. Another evidence showed that JAM-A, together with CD99 and CD31, forms a transient ring structure facilitating leukocyte transmigration[73]. The ring-like structures play an active role in organizing leukocytes transmigration.

JAM-A also play a role in angiogenesis. Naik and his colleagues found that basic fibroblast growth factor (bFGF)-induced angiogenesis depends on JAM-A/αvβ3 integrin complex through JAM-A signaling[162, 163]. In addition, JAM-A on $CD34^+$ stem cells mediates adhesion to the vessel wall after injury and differentiation into endothelial progenitor cells to facilitate re-endothelialization[164]. JAM-A is crucial for correct endothelial cell motility, directional movement and focal contact formation and $CD34^+$ stem cell differentiation, and thereby might be involved in angiogenesis in endothelial recovery after vascular injury.

Endothelial JAM-A is expressed in tight junction and re-localized to the apical surface under inflammatory conditions. Using a blocking monoclonal antibody against JAM-A on cultured endothelial cells inhibit monocyte transmigration[156]. Furthermore, treatment with a monoclonal antibody against JAM-A significantly inhibit leukocyte accumulation in cerebrospinal fluid and infiltration in brain parenchyma[165]. In atherosclerosis, endothelial JAM-A facilitates plaque formation. In early lesions of carotid arteries from apoe-deficient mice, endothelial JAM-A mRNA and protein expression increased[145], similar in human atherosclerotic plaque[158]. The increase of endothleial JAM-A in human atherosclerotic plaque also was validate by microarray technique[146], which might facilitate leukocytes infiltration and increase plaque volume. Recently, we demonstrated that JAM-A has a cell type-specific impact on atherosclerotic plaque formation. Whereas endothelial JAM-A promoted plaque formation by enhanced luminal availability under pro-atherosclerotic conditions, thus guiding monocytes to sites for plaque development.

The expression and luminal enrichment of JAM-A was exacerbated by disturbed flow conditions, as encountered at predilection sites, conversely, laminar shear flow induced microRNA-145 to repress JAM-A expression, indicating the contribution of limiting the susceptibility to atherosclerosis[40]. This gave a novel explanation of the higher susceptibility to atherosclerosis in regions with disturbed shear flow. Targeting endothelial JAM-A may serve as a feasible option to protect against vascular inflammation.

JAM-A on circulating leukocytes, including monocytes, lymphocytes, dendritic cells and neutrophils, mediates cells migration together with their integrins, but it shows cell-specific impact. On monocytes, not much is known about the specific roles of JAM-A. The only evidence is reduced monocyte adhesion to atherosclerotic endothelium of carotid artery perfused with monocytes pretreatment of soluble JAM-A.Fc in a VLA-4 independent manner[145]. On T cells, pretreatment of JAM-A antibody or soluble JAM-A.Fc in vitro assays reduced T cell arrest under flow conditions and trans-endothelial chemotaxis towards SDF-1[145]. However, JAM-A on polymorphonuclear cells reduced diapedesis with impaired de-adhesion and polarized motility in peritonitis and ischemia-reperfusion injury[166]. Recently, our group found that JAM-A deficient monocytes indeed displayed defects in de-adhesion and complete trans-endothelial migration, likely a result of persistently increased activity of β2 integrins, because monocytes were entrapped between the endothelial cell layer and the basement membrane and may thereby cause vascular damage. Leukocyte JAM-A might limit lesion formation by enabling physiological de-adhesion and migration of mononuclear cells[40].

On dendritic cells, JAM-A deficiency caused an increased random motility and transmigration across lymphatic ECs but not microvascular ECs. In vivo, JAM-A$^{-/-}$ mice showed enhanced DC migration to lymph nodes, whereas endothelial JAM-A deficiency did not alter dendritic cell transmigration[167], indicating a specific role of JAM-A in dendritic cell migration.

On neutrophils, JAM-A deficiency leads to defective uropod retraction and trans-endothelial migration in inflammatory models of peritonitis and heart ischemia/reperfusion as well as in adhesion and tans-migration assays[166]. Additionally, JAM-A$^{-/-}$ neutrophils adhered more efficiently to ECs and basement membrane proteins and their polarized movement was strongly reduced. Thus, JAM-A control neutrophil diapedesis through vessel wall. On activated neutrophils, JAM-A concentrated in a polarized fashion at the leading edge and uropod, while a significant amount of JAM-A is internalized and co-localized with integrin β1. JAM-A$^{-/-}$ neutrophils are unable to internalize integrin β1 upon chemotactic stimuli and this leads impaired uropod retraction and polarized cell motility. These phenotypes indicate that JAM-A is required for the internalization and recycling of integrins during neutrophil migration and might explain that the directional migration of JAM-A$^{-/-}$ neutrophils is impaired[168]. The small GTPase Rap-1 plays a role in integrin internalization and it´s activity is reduced in JAM-A$^{-/-}$ neutrophils[169]. These evidences indicate that JAM-A on neutrophils controls the regulated migration cascade combined with integrins.

Notably, the F11 receptor was found on the surface of human platelets and is a member of immunoglobulin superfamily[170]. A monoclonal antibody MabF11 against this membrane protein could cause platelet activation, such as platelet aggregation, granule secretion and phosphorylation of intracellular proteins[143]. In another study, this antibody activated platelets through cross-linking of JAM-A with FcγRII receptor on the membrane, triggering actin filament assembly with conversion of discoidal platelet to activated shapes and leading to platelet aggregation. The actin filament assembly is dependent on phosphoinositide-3 kinase activation[171]. Additionally, JAM-A was found to be phosphorylated in thrombin and collagen activated platelets, thus initially hinting towards a physiological role[172], despite the adhesion function. Thus, intensive investigation about JAM-A on platelets has occured in past years.

Human JAM-A cloned into Chinese hamster ovary (CHO) cells revealed that JAM-A accumulated on the cell surface and enhanced localization between two adjacent cells[170]. Furthermore, the intracellular tail of JAM-A was found to be co-localized with cortical f-

actin, but not with cell-spanning stress fibers. Interestingly, less platelet adhered to immobilized JAM-A$^{-/-}$ CHO cells compared with JAM-A expressing CHO cells, providing initial for cell–cell binding mediated by JAM-A hemophilic interactions[170]. Additionally, Babinska and colleagues found that platelets specifically adhered to a matrix made of immobilized JAM-A molecules. They also reported that platelets adhesion to cytokine-(TNF-α, INF-γ) activated HUVEC monolayers, is mainly mediated by homophilic interactions between JAM-A of platelets and EC[96]. Thus, JAM-A through homophilic interactions mediates platelet-platelet and platelet-endothelial cells adhesion.

Mounting evidence suggests that JAM-A regulates platelet activation, dependent on integrin αIIbβ3. Interestingly, JAM-A-deficiency was shown to cause platelet hyperreactivity. On resting platelets, the conformation of integrin αIIbβ3 under low-affinity is converted to a high affinity state after platelet activation, known as inside-out signaling[173]. Ligand binding to the activated integrin triggers cascade signaling events, named outside-in signaling[174]. Timely and rapid activation of integrin is pivotal for hemostasis, but unwanted activation can result in thrombosis[175]. However, the effects of unwanted or accidental activation of integrin in atherosclerosis, is unclear.

Integrin outside-in signaling for platelet activity begins to be characterized. The signal molecule c-Src binding with integrin, a member of the Src family kinase (SFKs), plays an important role in outside-in signaling[176]. In resting platelets, Src is kept in inactive state through two intramolecular interactions, including the binding of the Src homology 3 (SH3) domain to a polyproline type II helix and the other binding through its Src homology 2 (SH2) domain to a phosphotyrosine residue (Y^{529}) in the C-terminal regulatory domain[177]. Maintaining SFKs in an inactive state requires phosphorylation of the C-terminal inhibitory Y^{529} residue by cytoplasmic kinases Csk (C-terminal Src kinase) or Csk homologous kinase. SFKs dephosphorylation can trigger integrin outside-in signaling. However, Csk should be recruited nearly to sites of Src to suppress Src activity. Thus, the molecule, which recruits Csk to Src, becomes very important. It has been found that JAM-A binding Csk forms complex with integrin, so JAM-A as the linker between Csk and Src to form a complex (Csk-JAM-A- integrin αIIbβ3-Src) maintains Src Y^{529}

phosphorylation in an inactive state[178]. After platelet activation, JAM-A detaches with Csk and is rapidly phosphorylated by PKC[179], followed by the complex disruption. Losing CSK, Scr Y^{529} is dephosphorylated and triggers downstream signaling. However, the signaling pathway still need more research, such as Scr activity after detaching from the complex.

Like other cell adhesion molecules (CAMs) as negative regulator for platelet activation[180], JAM-A as a negative regulator controls platelet activity, such as protection from thrombosis[181]. JAM-A negatively regulates platelet function in vivo, which was certified by JAM-A–deficient mice undergoing a pro-thrombotic phenotype. Additionally, transplantation of JAM-A–deficient platelets into wild-type background also displays a pro-thrombotic phenotype. In these mice, JAM-A–deficient platelets hyper-aggregate in response to a low dose of physiologic agonists, are named hyperreactivity. This hyperreactivity of JAM-A–deficient platelets is not due to augment of inside-out signaling, such as granular secretion, Thromboxane A2 (TxA2) generation and integrin activity, but due to the enhanced outside-in signaling as increased αIIbβ3 phosphorylation[181, 182].

As more evidencs proposed that platelets are initiators and propagators of atherosclerosis[60] and JAM-A might regulate platelet function during thrombosis and atherosclerosis[96, 171, 183]. Now we believed that platelet JAM-A displays two distinct functions. First, JAM-A mediates integrin αIIbβ3 dependent platelet activation. Second, JAM-A mediates platelet adhesion independent of integrins or fibrinogen receptors via homophilic JAM-A interactions with other cell types, thus playing a role in physiological processes important for platelet aggregation. But little is known the detailed functions about JAM-A regulating platelet in atherosclerosis.

2 The aims of this study

Background: Platelets have an essential role in hemostasis and also act as immune cells to spark vascular inflammation. As the first circulating cells adhering to atherosclerotic predilection sites and to injured vessel wall, platelets promote leukocyte migration into vessel wall, which is the main driving force of vascular inflammation and remodeling. Platelets can bridge leukocyte adhesion to endothelium and also secrete cytokines for leukocytes recruitment. Thus, platelets are implicated as initiator and propagator of vascular remodeling. Although loss of platelet function reduces atherosclerotic plaque formation in hyperlipidemic mice[60, 77], in fact, very few studies can address whether platelet hyperreactivity augments vascular inflammation and remodeling or not.

JAM-A as an adhesion molecule regulate endothelial permeability, platelet activity and leukocytes migration. But it shows cell-specific roles in different cell types. Somatic deficiency of JAM-A led to decreased neointimal formation, but no effect on plaque formation. JAM-A on endothelial cells and leukocytes appears to have opposite roles for plaque formation[40]. Thus, it is worthwhile to investigate functions of platelet JAM-A in vascular remodeling. Furthermore, JAM-A inhibits platelet activity through outside-in signaling of integrin αIIbβ3. Deletion of JAM-A in platelets caused hyperreactivity[181, 182], but whether a lower activation threshold of platelets leads to platelet-mediated increase of vascular inflammation in vivo needs to be investigated. In addition, the molecular basis for the regulation of integrin signaling by JAM-A is not yet fully characterized.

The aims of this study were:

1. To find out whether trJAM-A–deficiency affects atherosclerotic development and neointimal formation after vascular injury in hyperlipidemic mice.
2. To determine how trJAM-A–deficiency causes platelet hyperreactivity.
3. To investigate the interactions among JAM-A-deficient platelets, leukocytes and inflammatory endothelium in the context of vascular inflammation.
4. To find out how platelet hypereactivity affects chemokine secretion.
5. To elaborate the signaling cascade of JAM-A regulating platelet activity through integrin αIIbβ3.

3 Materials and Methods

The general chemicals were purchased from Fluka (Buchs, Switzerland), Sigma (Deisenhofen, Germany) or Roth (Karlsruhe, Germany) in analytical grade quality, unless indicated otherwise. All reagents, buffers and solution were prepared with double distilled water (Heraeus Destamat, Heraeus, Germany) or Millipore water (Milli-Q Plus ultrapure purification, Millipore, MA). All protocols were adapted from standard protocols unless otherwise described.

3.1 Materials

3.1.1 Instruments

Equipment	Manufacturer
Balance	Analytical Plus, Ohaus, Pine Brook, USA
Centrifuge	Centrifuge5415 R, Eppendof Heraeus®Multifuge®3S-R, Thermo Scientific
Digital camera	Leica DFC365 FX, Leica Microsystems, Germany
Embedding machine	Leica EG1160, Leica biosystems, Germany
Flow cytometers	FACS Canto II, BD Biosciences, San Jose, CA, USA
Hematology analyzer	ScilVet ABC, scil Animal Care
Hematology analyzer	ScilVet ABC, scil Animal Care
Microscope	Leica DM RBE, Leica Microsystems, Germany
Multiplate® platelet analyzer	Roche Diagnostics, Mannheim, Germany
Microplate reader	GENios,Tecan, Männedorf, Switzerland
PH-meter	InoLab level 1,WTW, Weilheim, Germany
Stereomicroscope	Leica S6, Leica Microsystems, Germany Stemi:2000-c, Zeiss
Tissue Processor(dehydration)	Leica ASP200 S, Leica biosystems, Germany

3.1.2 Reagents and materials

General Reagents and materials	Catalog number	Manufacturer
4% buffered formaldehyde	P087.3	Carl Roth
4'.6-diamidino-2-phenylindole(DAPI)	H-1500	Vector Labs
Adenosine diphosphate (ADP)	50704	Biopool
Antigen retrieval solution	S1699	Dako
Antagonist(antiamzolhydrochlorid)	400936	Cp-pharma
Bovine serum albumin (BSA)	11922	SERVA
CCL5/RANTES ELISA Kit	DY478	R&D Systems
Cholesterol-CHOD-PAP kit	12016630122	Cobas
Citrate-coated tubes	352052	BD Falcon
CXCL4/CXCL4 ELISA Kit	DY595	R&D Systems
DC protein assay kit	500-0111	Bio-Rad
Dimethyl sulfoxide (DMSO)	A994.1	Roth
EDTA-coated tubes	41.1504.005	Sarstedt
Elastica van Gieson (EVG) staining kit	12739	Morphisto
Ethanol	9065.3	Roth
FACS tubes	352052	BD Falcon
Fixation/permeabilization solution kit	554714	BD Bioscience
Fibrinogen	CT15	Oxford Biomedical Research
Goat serum	X0907	Dako
Hank's Balanced Salt Solution (HBSS)	14065-049	Life Technologies
Isopropanol	109634	Merck millipore
KB SRC4 (Src inhibitor)	4660	Tocris
Ketamine	618926	pfizer
2x Laemmli Sample Buffer	161-0737	Bio-Rad
Medetomidine	45081	Pfizer
Oil Red O	O0625-100G	Sigma Aldrich
PP2 (Src inhibitor)	529573	Merck millipore

Protein G-coupled magnetic beads	10003D	Life Technologies
PTP inhibitor IV	540211-10MG	Merck millipore
PTP1B inhibitor	539741	Merck millipore
SHP1/2 PTPase inhibitor (NSC-87877)	565851	Merck millipore
SU6656 (Src inhibitor)	S9692	Merck millipore
Surgical suture 7/0	IO 051391	Serag Wiessner
Suture clips	BN507R	Aesculap
TBS-Roti-block solution	A151.1	Carl Roth
Tirofiban	Aggrastat®	MSD
Thrombin	T7513	Sigma Aldrich
Triglycerides-GPO-PAP kit	11488872216	Cobas
Tween® 20	P1379	Sigma-Aldrich
Xylol/Xylenes	582698	Sigma Aldrich
Tris-(hydroxymethyl)-aminomethan	2449.3	Carl Roth
12-Well Plate	3512	Coring

3.1.3 Antibodies

Main Antibodies	Clone	Manufacturer
anti-CD41 PE	558040	BD Pharmingen
anti-CD41 FITC	MWReg30	eBioscience
anti-CD31 eFluor®450	390	eBioscience
anti-CD45 eFluor®450	30-F11	eBioscience
anti-CD115 PE	AFS98	eBioscience
anti-ESAM	AB4319	Merck Millipore
Anti-Von Willebrand Factor	A0082	Dako
anti-mouse IgG DyLight 550	ab96880	Abcam
anti-JAM-A AF488	H202-106	AbD Serotec
anti-JAM-C PE	FAB7050P	R&D systems
anti-human/mouse CD3	A0452	Dako
anti-human/mouse SMA	1A4	Dako

anti-Ly6G PerCP-Cyanine 5.5	RB6-8C5	eBioscience
anti-mouse MAC-2	CL8942AP	Cedarlane
anti-mouse phosphp-Src (Y418) eFluor660	50-9034-41	eBioscience
anti-phosphotyrosine	4G10	Merck Millipore
anti-rat IgG DyLight 488	ab96887	Abcam
anti-rabbit IgG DyLight 650	ab96926	Abcam

3.2 Methods

3.2.1 Mouse models

Mice carrying cre-recombinase under the control of platelet factor 4 (PF4)-promoter were a gift from Dr. R.C. Skoda, University Hospital Basel[184] and were backcrossed in an apolipoprotein e (apoe)-deficient background (C57Bl/6) at least ten generations. These mice were crossed with JAM-A$^{flox/flox}$apoe$^{-/-}$ mice to obtain platelet-specific (tr)JAM-A$^{-/-}$apoe$^{-/-}$ mice[40]. Littermates not containing the PF4-cre transgene were used as (tr)JAM-A$^{+/+}$apoe$^{-/-}$ controls. All animal experiments were approved by local authorities (Regierung von Oberbayern, Munich, Germany). In atherosclerosis research, mice were put on high fat diet (HFD, 21% fat, 19.5 % casein, 0.15% cholesterol, ssniff, Soest, Germany) for 2, 6 or 12 weeks. For neointimal formation research, mice were put on HFD for 1 week before and 2 weeks or 4 weeks after wire-injury operation.

3.2.2 Wire-injury in mouse carotid artery

The wire-injury in carotid artery of trJAM-A$^{+/+}$apoe$^{-/-}$ and trJAM-A$^{-/-}$apoe$^{-/-}$ mice is to denudate endothelium completely without internal lamina broken[38]. After mouse anesthesia (ketamine 80 mg/kg and medetomidine 0.3 mg/kg), ointment is applied to the eyes to prevent cornea drying. The mouse was fixed on a heating plate (tempcontrol 37-2 digital) at 37 ˚C. Following midline neck incision, the left carotid artery and branches were exposed under Stereomicroscope (Stemi: 2000-c linked with the KL 1500 LCD; Zeiss). The left common carotid artery and internal and external carotid arteries were ligated proximally using 7/0 surgical suture (Serag Wiessner, IO 051391) without tying off. Proper suture tension is just for interrupting blood flow. Additional suture was added

around distal external carotid artery near bifurcation without tying off. After crosscutting a very small hole on external artery between those 2 sutures around the external artery, a flexible angioplasty guide-wire with 0.36mm diameter was inserted into common carotid artery and advanced about 1 cm. Complete and uniform endothelial denudation was achieved by 3 passes back and forth along common carotid artery and with 3 times of rotating motion. The wire must move freely and should not get caught at a hole in the vessel during motion. After injury, the proximal and distal sutures around external carotid artery were tied off and other sutures were moved out gently. The blood flow recovered to flow across the carotid artery. To check carotid artery whether clotting, the common carotid artery can be monitored for pulsation and color of blood. The skin incision was closed by two suture clips (Aesculap, BN507R). After operation, mice were injected antagonist (antiamzolhydrochlorid, 0.03mg/kg) to recovery and then put on HFD for 2 weeks or 4 weeks.

3.2.3 Mouse blood collection and analysis

Mice were anesthetized and blood was retro-orbitally collected into EDTA-coated tubes (sarstedt, 41.1504.005) for cell counts, plasma isolation, flow cytometry or two-photon laser scanning microscopy experiment. Blood was collected into citrate-coated tubes (sarstedt, 41.1506.005) for platelet function assessment (platelet aggregation assay). Cell counts and mean platelet volume (MPV) were measured using an automated hematology analyzer (scil Animal Care).

3.2.3.1 Leukocytes isolation

Mice blood was taken from retro-orbital plexus into citrate-coated tubes and centrifuged at 1600 rpm for 10 minutes with break (Eppendof, Centrifuge 5415 R). The supernatant was taken out without any erythrocytes, just yellow supernatant for platelet isolation. 1ml erythrocytes-lysis buffer (8.4g NH_4Cl, 0.84g $NaHCO_3$, 1L H20, pH=7.2-7.6, RT) was added to the rest (all other cells in the pellets) and then transferred into labeled FACS tubes (BD Falcon, Cat.352052) with additional 2 ml erythrocytes-lysis buffer. After incubation for 5 min, the sample was centrifuged at 300 g for 5 minutes with break (Thermo Scientific, Heraeus® Multifuge® 3S-R Centrifuge). The supernatant was

dumped. The pellets were re-suspended in new 3 ml erythrocytes-lysis buffer. These lysis steps were repeated twice until pellet turns white. When all the erythrocytes are lysed, the pellets are peripheral blood mononuclear cells (PBMCs) for flow cytometry.

3.2.3.2 Platelet isolation

Mice platelets were isolated and washed by centrifugation as described[185]. The supernatant separated with leukocytes above was centrifuged at 1000 rpm for 6 minutes without break (Thermo Scientific, Heraeus® Multifuge® 3S-R Centrifuge). The supernatant (Platelet Rich Plasma, PRP) was saved and added citrate buffer (80mM sodium citrate tribasic, 52mM citric acid, 183mM glucose, filled and stored at 4˚C). The working volume of citrate buffer is 1/25 (v/v) of whole solution volume. Then, the sample was centrifuged at 5600 rpm for 5 minutes with break. The supernatant was discarded and the pellet was re-suspended in 200µl PBS with 1/25 citriate buffer for platelet experiments.

3.2.3.3 Plasma isolation

Mice blood was taken from retro-orbital plexus into EDTA-coated tubes and centrifuged at 1500 rpm for 15 min. The supernatant (platelet-rich-plasma, PRP) was taken out and centrifuged again at 13000 rpm or 5 min. The supernatant (platelet-poor-plasma, PPP) was stored in -20˚C.

3.2.4 Mouse organ samples collection and process

1) **Dissection:**

   Mice were anesthetized and blood was retro-orbitally collected in citrate-coated tubes. Dissection was done under stereomicroscope (Leica S6) to expose mice hearts and vasculature. A catheter was inserted into left ventricle and linked a syringe to perfuse cold PBS and cold 4% buffered formaldehyde (PFA, Carl Roth, Karlsruhe, Germany), as least 5ml, into mouse circulation. Exclusively, if the artery is prepared for protein, DNA or RNA extraction, only cold PBS is used without PFA.

2) **Fixation:**

   If necessary, the aorta and carotid arteries were carefully excised without peripheral tissues, especially fat tissue. Heart into Eppendorf ® Tube, carotid arteries straightly in a small tissue cassette coated inside with paper tissue and aorta on a plastic plate using needles to fix, all were put overnight in 4% PFA.

3) **Dehydration and embedding:**

   After fixation, the hearts were cut off in the middle and put in tissue cassettes. Then, hearts and carotid arteries were placed into histokinate (Leica, ASP200S) for dehydration procedure automatically following dehydration solutions 70%, 70%, 96%, 96%, 100%, 100% to 100% ethanol. Then, the samples were immersed in xylene and covered with paraffin. At last, samples were embedded in embedding machine (Leica, EG1160) to form paraffin blocks. Carotid arteries should be kept standing up straightly in a mental chamber full with melted wax. Heart should be put the apical side to the bottom of the mental chamber.

4) **Tissue serial sections:**

   The paraffin blocks were cut in sectioning machine (Leica, RM2155). The aortic root with 3 valves up 300µm length was cut into 4µm transverse section as each piece and left common carotid artery up to 1cm from bifurcation was cut and prepared for histology analysis.

3.2.5 Histological staining

3.2.5.1 EVG staining

Elastica van Gieson (EVG) staining kit (Morphisto, Art.-Nr.12739) is used to demonstrate elastic fibers in tissue sections for plaque or neointimal area quantification. The plaques in aorta roots and neointima in injured carotid arteries were visualized and quantified by Leica Qwin Imaging software (Leica Microsystems, Wetzlar, Germany). The paraffin sections were chosen out intermittently from each mouse and were submerged in the following solutions for de-paraffinization: xylol for 15 minutes in twice, ethanol for 5 minutes twice, 96% ethanol for 5 minutes, 70% ethanol 5 minutes and then washed in PBS for 5 minutes. The deparaffinized sections were then stained in the

following solutions: incubated in Resorcin-Fuchsin (staining elastic fibers) for 20 minutes, rinsed for 1 minute in running tap water, incubated for 20 minutes in Weigert's iron hematoxylin solution (staining nuclei), rinsed for 10 minutes in running tap water, put in 1% Hydrogen chloride/70% ethanol solution for 10 seconds (color differentiation), washed for 5 seconds in water, incubated in Van Gieson's Solution for 1 minute (staining connective tissue), washed in water for 5 seconds, and then incubated in 96% ethanol for 5 minutes in twice, Isopropanol for 2 minutes, xylol for 5 minutes in twice. The sections were covered with mounting solution (Roti-HistoKit II, Carl Roth GmbH) and pictures were taken under microscopy (Leica DM RBE). The areas for plaque and aortic root, carotid artery lumen and internal and external elastic lamina were measured by Lica software (Leica Application Suit V4).

3.2.5.2 Aortic oil-red-o staining

The oil-red-o staining was used to quantify aortic plaque in en face. The oil-red-o stocking solution was prepared as 1g oil-red-o powder dissolved in 200ml 99% isopropanol. The working solution was made up freshly from the stock solution each time: 75ml stocking solution was diluted with 50ml distilled water and stood for 1 hour, then filtered with filter paper.

After dissection of complete aorta, from aortic root to iliac branch, the aorta is longitudinally opened and stapled on a black plastic plate by small needles in 4% PFA for overnight. In the next day, aortic adventitial tissue was striped gently and completely. Then, the aorta was dipped in 60% isopropanol for 10 times. After that, aorta was incubated in the oil-red-o working solution for 15 min. Then, the excess oil-red-o solution was removed by dipping the sections 10 times in 60% 2-propanol. The aorta was moved onto glass slide and mounted with glycerol (Cat.3783.5, Carl Roth). The pictures were taken in a series from a complete aorta under 2.5x objective magnification of microscope (Leica DM RBE). The pictures of different parts of the aorta were merged to construct en face aorta using photoshop software. The percentage of plaque in whole aorta area was quantified using by Lica Microsystems (Leica Application Suit V4).

3.2.5.3 Immunofluorescence staining

Immunofluorescence (IF) staining was used to test JAM-A, CXCL4 and CCL5 expression in platelets and cell composition in plaque and neointima, such as $MAC2^+$ macrophage, $CD3^+$ T cell, α-SMA^+ smooth muscle cell (SMC) and von Willebrand Factor (vWF) staining.

IF staining of platelet

Isolated platelets as described[185] from mice blood was fixed and permeabilized with 500µl Cytofix/Cytoperm (BD Bioscience, 554714) for 20 minutes. After centrifuged 5 minutes at 2200rpm, the pellet was re-suspended in 250 µl 1x Perm/Wash Buffer (BD Bioscience, 554714). This washing step was repeated. Primary Antibodies, anti-CXCL4 (R&D Systems, Minneapolis, MN) and anti-CCL5 antibodies (both R&D Systems, Minneapolis, MN), were incubated for 2 hours at room temperature in the dark. Then, washing step was followed. Secondary antibodies conjugated with DyLight®-488, -550 or -650 (all Abcam, Cambridge, UK) were incubated for 1.5 hours at room temperature in the dark. After washed twice, the pellet was re-suspended in 100µl PBS. 20 µl of platelet suspension was mounted on the glass slide with Vectashield mounting medium (Vector Labs). The pictures are taken under fluorescence microscope (Leica DM 6000) with a monochrome digital camera (Leica DFC365 FX).

IF staining of plaque and neointima

The sections of aortic root and injured carotid arteries were deparaffinized as the same as in EVG staining protocol. Then, heat-induced antigen retrieval was done. The samples were boiled at 180 watt in 1 x target retrieval solution (Dako, S1699) by microwave and then cooled down for at least 20 minutes at room temperature. After antigen retrieval, the samples were put in distilled water for 5 minutes, then in PBS for 5 minutes. The liquid around sections was removed on the slide very carefully with a paper towel without touching the sections (the sections were kept moisture in the whole procedure). Then, blocking non-specific binding to the antibodies, for example, the secondary antibody was produced from goat, so the blocking solution was 10% goat serum (Dako, X0907) diluted in PBS and 50µl of the blocking solution was put on each sample for 20 minutes at room temperature. Then, the liquid around samples was removed as before. The first antibodies

cocktail were incubated with samples. In this experiment, rat anti-mouse MAC-2 (2.5µg/ml, CL8942AP, Cedarlane) and rabbit anti-human CD3 (2.7µg/ml, A0452, Dako) were diluted in 0.5 % goat serum/PBS and added on the samples for incubation over night at 4°C in humid chamber. On the next day, samples were washed in PBS 3 times. Then, incubation with secondary antibodies cocktail: the liquid around was removed. In this experiment, goat anti-rat IgG DyLight 488 (5µg/ml, ab96887, Abcam) and sheep anti-rabbit DyLight 650 (5µg/ml, ab96887, Abcam) were diluted in 0.5% goat serum/PBS and incubate for 45 min at room temperature in dark. For smooth muscle cells staining, the antibodies are mouse anti-human smooth muscle actin (1:100 dilution, clone 1A4, Dako) incubated for 1 hour and goat anti-mouse IgG DyLight 550 (5µg/ml, ab96880, Abcam) incubated for 1 hour. The same procedure is used in von Willebrand Factor staining (1:600, Dako, A0082). At last, after washing in PBS for 5 minutes, sections were mounted with Vectashield 4'.6-diamidino-2-phenylindole (DAPI, Hard Set Vector Labs) and covered by cover slides. Images were taken under fluorescence microscope (Leica DM6000) with the camera (Leica DFC365 FX). The quantification was done by Leica Microsystems (Leica Application Suit V4). For background assessment, samples were stained instead of primary antibodies with appropriate IgG, followed by the secondary antibodies.

3.2.6 Multiplate® platelet aggregation measurement

Platelet aggregation in response to adenosine diphosphate (ADP, biopool) and thrombin (Sigma Aldrich) was assessed using mouse whole blood within 2 hours after isolation. Multiple electrode aggregometry technology in a Multiplate® platelet analyzer was according to manufacturer's instructions (Roche Diagnostics, Mannheim, Germany)[186].

Blood was collected into EDTA-coated tubes. The multiplate machine was switched on and warmed up in 20-30 minutes. Saline solution must be preheated for at least 10 minutes. The test cells were inserted into the measuring position and attached to sensor cables. 150µl 0.9% saline and 150µl blood were added in to the test cell. In some experiments, platelets were pretreated with αIIbβ3 integrin antagonist tirofiban (Aggrastat®, MSD, 1µg/ml) or the Src kinase inhibitor PP2, SU6656 and KB Src 4 (Merck Millipore or Tocris, 20, 2.5 and 0.09 µmol/l respectively). The incubation time

was 3 minutes. After incubation, the stimulus, such as ADP (0.5µm/l, 1 µm/l, 2µm/l respectively) or thrombin (0.021U/ml, 0.11U/ml, 0.51U/ml, 1U/ml respectively), was added into the bottom of plastic cells and the test was started. Then, the process of platelet aggregation was recorded as curve in image. Platelet aggregation was presented as area under the curve in aggregation units (AU), expressed as AU*min, which was quantified.

3.2.7 Protein extraction

The mice platelets pellets were lysed on ice using the lysis buffer: 50mM tris pH7.4, 150mM NaCl, 5mM EDTA, 0.1mM DTT, 1% NP-40, 1% CHAPS.

Protein concentrations of platelet lysis were determined by Bio-Rad protein assay.

3.2.8 Immunoprecipitation

Immunoprcipitation was used for testing trJAM-A phosphorylation and related kinase involved in trJAM-A de-phosphorylation after platelet stimulation by fibrinogen. Isolated washed platelets from mice were incubated on immobilized heat-inactivated Bovine serum albumin (BSA, 5mg/ml, SERVA 11922) or mouse fibrinogen (0.1mg/mL, Oxford Biomedical Research) for 90 minutes at 37°C, respectively as described[178, 187].

The platelets were stimulated on immobilized fibrinogen. The fibrinogen-coated wells (Costar®12 Well Plates, Corning, 3512) were prepared for stimulating platelets. Mouse fibrinogen (1mg/ml stock solution, -80°C, Oxford Biomedical Research) is diluted in 0.9% NaCl solution (1:10, working concentration 100µg/ml) and then poured into the wells to cover the bottom (about 400µl/well). The control group used 0.5% heat-inactivated BSA. After incubation at 37°C for 1 hour, the fibrinogen solution was dropped out and 0.5% inactivated BSA blocking solution was added into the wells for 20 minutes at 37°C. Note: 0.5% BSA working solution should be inactivated previously at 56°C for 45 minutes in water bath.

The isolated platelets was re-suspended in 1/25 (v/v) citrate buffer and measured in the automated hematology analyzer (scil Animal Care). Then, platelets were diluted in 1/25 (v/v) citrate buffer to the concentration 1x 10^8/ml. In some experiments, protein tyrosine

phosphatase inhibitors: IV (20µmol/L), XXXI (30µmol/L), NSC-87877 (0.7 or 10µmol/L) and PTP1B inhibitor (8µmol/L), or vehicle (DMSO) were mixed with platelet suspension and then pipetted into fibrinogen- or inactivated BSA coated wells during adhesion process. The platelets were incubated for 90 minutes at 37°C (cover with the lid) and lysed using platelet lysis buffer, as described above.

JAM-A was immunoprecipitated from platelet lysis, by specific antibody (clone H2O2-106, AbD Serotec) and protein G-coupled magnetic beads (Dynabeads, Life Technologies). It analyzed in western blotting.

Dynabeads® Protein G binds antibody via their Fc region and was separated using a Dynal® magnet. In this experiment, 5µg anti-mouse JAM-A rat monoclonal was diluted in 200µl PBS/0.05% Tween 20, with the beads for 1hour. After washing in 200µl PBS/0.05% Tween 20, platelet lysis (protein amount 25µg) was added and incubated with rotation for 2 hours at room temperature to allow JAM-A to bind to beads-antibody complex. After washing bead-Ab-Ag complex three times using 200 µl PBS each time, 20µl 2x Laemmli Sample Buffer (Bio-Rad, 161-0737) was added to the bead-Ab-Ag complex and denatured by heating for 10 minutes at 95˚C. After loading samples, western blotting was done. After transferring the proteins to nitrocellulose membrane, the primary antibody: anti-phosphotyrosine mouse monoclonal (clone 4G10, Merck Millipore) and HRP-conjugated secondary antibody were incubated. After detecting protein bands, the anti-phosphotyrosine antibody and secondary antibody were stripped from the membrane by the Restore Western Blot Stripping Buffer (Thermo Scientific, 46430) for 15 minutes at room temperature. After blocking the membrane again, anti-mouse JAM-A rat monoclonal antibody (clone H2O2-106, AbD Serotec, Düsseldorf, Germany) was incubated to perform next western blotting steps. This step was to detect the immunoprecipitated protein (JAM-A).

3.2.9 Enzyme-linked immunosorbent assays (ELISA)

ELISA is used for testing concentrations of cytokines, chemokines and other antigens that are secreted or released into plasma from cells. The coupled antibodies with easily-assayed enzyme produce a colored substrate indicating a positive reaction.

Concentrations of chemokines CXCL4 and CCL5 were measured in platelet poor plasma (PPP) from mice fed a HFD for 2, 6 and 12 weeks using Enzyme-linked immunosorbent assays (ELISA) kits (both R&D Systems, Minneapolis) accordance with the manufacturer´s instructions. The capture antibody specific for the antigen of interest is pre-coated onto a 96-well microplate and incubated over night at RT. After aspiration and washing 3 times using the 0.05% Tween 20 in PBS, plate was blocked with 1% BSA in PBS for 1 h. After washing, the standards or samples were added for binding to capture antibody and incubated for 2 h. Following the wash, the detection antibody was added and incubated for 2 h at room temperature. After washing, the streptavidin-HRP was added and incubated for 20 minutes. Avoid placing the plate in direct light. Last, when the substrate solution was added and incubated at room temperature, the reaction yielded blue then turns to yellow. After 20 minutes, the stop solution (2N H_2SO_4) was added. The optical density was determined using a microplate reader set to 450 nm/570 nm. A standard curve was generated for each set of assayed samples and concentrations of the samples were calculated according to standard curve.

3.2.10 Enzymatic colorimetric method

Total cholesterol and triglycerides concentrations were analyzed using enzymatic colorimetric method (Cholesterol-CHOD-PAP kit and Triglycerides-GPO-PAP kit, both Roche Diagnostics) according to the manufacturer's instructions.

Prepare the standard, which can be used for cholesterol and triglycerides tests. The stock concentration of the standard for cholesterol and triglycerides is 4.35 and 1.44 mm/L, respectively. A standard curve was created using 2-fold serial dilutions in 0.9% NaCl. Dilute the samples (if necessary) in 0.9% NaCl. Add 50µl of standard or samples and 150µl reactant reagent per well. Incubate 30 minutes at 37˚C in dark. Cool down for 5 minutes at RT and microplate reader set to 510 nm.

3.2.11 Flow Cytometry

The laser based flow cytometry is a biophysical technology for analyzing cells by their size, granularity and allows for detection of protein expression. In a fluid sheath stream, one cell at a time passes by an argon laser, which excites fluorescently labeled cells.

Measurements of the size (forward scatter) and granularity (sideward scatter) are independent from fluorescence signals.

In this study, the flow cytometry technique was utilized for testing platelet JAM-A, JAM-C and ESAM expression and counting complex formation in vitro of platelets with monocytes or neutrophils. Phosphorylated c-Src was detected using an eFluor®660-conjugated mouse monoclonal antibody, specific for mouse/human Src phosphorylation at tyrosine 418 residue (Y418) (eBioscience). For assessment of JAM-A expression in platelets, platelets were labeled with anti-JAM-A AF488-conjugated (AbD Serotec) and anti-CD41 PE-conjugated (BD Pharmingen) antibodies. For the platelet-leukocyte interactions, isolated platelets were activated by 0.5U/ml thrombin and added to isolated erythrocyte-free leukocytes for 20 minutes at 37°C. Finally, cells were stained with anti-CD41 FITC-conjugated (eBioscience), anti-CD45 eFluor®450-conjugated (eBioscience), anti-CD115 PE-Cyanine 7-conjugated (eBioscience) and anti-Ly6G PerCP-Cyanine 5.5-conjugated (eBioscience) antibodies. In some experiments, platelet-leukocyte interactions were analyzed after pretreatment of the platelets with αIIbβ3 integrin antagonist tirofiban (Aggrastat®, MSD, 1µg/mL). For the adhesion molecules expression, isolated platelets were stained with anti-JAM-A AF488-conjugated (AbD Serotec), anti-JAM-C PE-conjugated and anti-endothelial cell-selective adhesion molecule (ESAM, Merck Millipore), incubated with DyLight®-650-conjugated secondary antibody (Abcam, Cambridge, UK). Samples were acquired by flow cytometry (FACSCantoII, BD Biosciences) after appropriate compensation settings and analyzed by FlowJo v.10 software (Tree Star Inc.).

3.2.12 Two-photon laser scanning microscopy

Two-photon laser scanning microscopy (TPLSM) is a fluorescence imaging technique with deep tissue penetration, efficient light detection and reduced tissue damage[188]. TPLSM imaging was performed using a scanner Leica SP5MP (Mannheim, Germany) with a pulsed Ti-Sapphire laser (MaiTai HP, Spectra Physics, Mountain View, USA) tuned to 800 nm and a 20×NA1.00 water dipping objective (Leica). Emitted fluorescence signals were detected using photo-multiplier tubes (PMTs). Image processing was

performed using LAS software (Leica) and Image Pro Analyzer v7.0 software (Media Cybernetics, Rockville, MD).

TPLSM was used to analyze platelet adhesion to atherogenic carotid artery wall. The trJAM-$A^{+/+}$apoe$^{-/-}$ and trJAM-$A^{-/-}$apoe$^{-/-}$ mice were fed a HFD for 2 weeks. Whole blood was retro-orbitally taken from the mice into citrate coated tubes and diluted with citrate buffer to adjust platelet count to 1×10^8 platelets/ml. Carotid arteries were carefully explanted, mounted in a customized perfusion chamber and pressurized at physiological pressure of 60-80 mmHg. Prior to perfusion, the endothelium was labeled with anti-CD31 eFluor®450-conjugated antibody (3.5µg/ml, eBioscience) and diluted blood was incubated with anti-CD41 FITC-conjugated antibody (3.7µg/ml, eBioscience) for 30 minutes. Then, blood was perfused along the mounted and pressurized vessel for 10 minutes at 0.5ml/min. In a second set of experiments, diluted blood was pretreated with integrin αIIbβ3 antagonist tirofiban (Aggrastat®, MSD, 1µg/mL) prior to perfusion. The first 500µm close to mounting pipettes were excluded due to possible handling damage. The corresponding wavelengths was using an acousto-optical beam splitter: 400-440 nm for second-harmonic generation (SHG); 460-490 nm (eFluor® 450) and 510-560 nm (FITC). Adherent platelets were recorded by TPLSM and counted per field of view of Z-stack maximum projections (FOV=240µm^2; voxel size: 0.23 x 0.23 x 1µm^3). Z-stacks were acquired at 0.1 Hz including two-fold frame averaging.

TPLSM was used to analyze platelet adhesion to wire-injured carotid artery. The trJAM-$A^{+/+}$ apoe$^{-/-}$ and trJAM-$A^{-/-}$apoe$^{-/-}$ mice were fed a HFD for 1 weeks. Then, the wire-injury operation was carried out on left carotid artery as described above. One hour after wire injury, the injured carotid artery was carefully explanted, mounted in a customized perfusion chamber and pressurized at physiological pressure of 60-80 mmHg. To detect whether endothelium denudation complete, endothelium was labeled with anti-CD31 eFlour488 or 450 (3.5µg/ml, eBioscience), and platelet was stained by CD41-PE (3.5µg/ml, BD Pharmingen) for 30 minutes. TPLSM was performed using a Leica SP5MP system. Emitted fluorescent signals were detected by PMTs tuned for the corresponding wavelengths using an acousto-optical beam splitter: 400-440nm for second-harmonic generation (SHG); 460-490nm (eFluor® 450), 510-540nm (Alexa

Fluor®488) and 570-600nm (PE). Z-stacks were acquired at 0.1 Hz over time including two-fold line averaging; One field of view (FOV=330µm2; voxel size: 0.32 x 0.32 x 1μm^3) was recorded. All pictures were processed using LAS software (Leica) and Image Pro Analyzer v 7.0 software (Media Cybernetics, Rockville, MD). More than 3 FOVs without endothelium signals in each carotid artery were taken for platelet quantification. The software (Lecia Application Suite X) constructed 3-D pictures for platelet volume measurement and automatically calculate the volume of platelet positive signal.

4 Results

4.1 Adhesion molecule expression in the vasculature

To investigate the Junctional adhesion molecule A (JAM-A) expression in cardiovascular system, we implemented trJAM-A$^{-/-}$apoe$^{-/-}$ mice and compared them to their control littermates (trJAM-A$^{+/+}$ apoe$^{-/-}$). The results showed that platelet-specific JAM-A was knockout (trJAM-A$^{-/-}$).

4.1.1 JAM-A deletion on platelets

Immunofluorescence staining shows CD41 as a marker of platelets and JAM-A expressed on isolated platelets from trJAM-A$^{+/+}$apoe$^{-/-}$ mice, but only CD41, not JAM-A, detectable on trJAM-A$^{-/-}$apoe$^{-/-}$ mice (Figure 5A). In line, flow cytometry showed that CD41 was expressed on isolated platelets from trJAM-A$^{+/+}$apoe$^{-/-}$ mice and trJAM-A$^{-/-}$ apoe$^{-/-}$ mice, but JAM-A was not detectable on platelets from trJAM-A$^{-/-}$apoe$^{-/-}$ mice (Figure 5B). The percentages of positive platelets expressing JAM-A were depicted in upper right quadrant of graphs, which is almost zero from trJAM-A$^{-/-}$apoe$^{-/-}$ mice, displaying genetic deletion of JAM-A. These data illustrate efficient knockout of trJAM-A in trJAM-A$^{-/-}$apoe$^{-/-}$ mice.

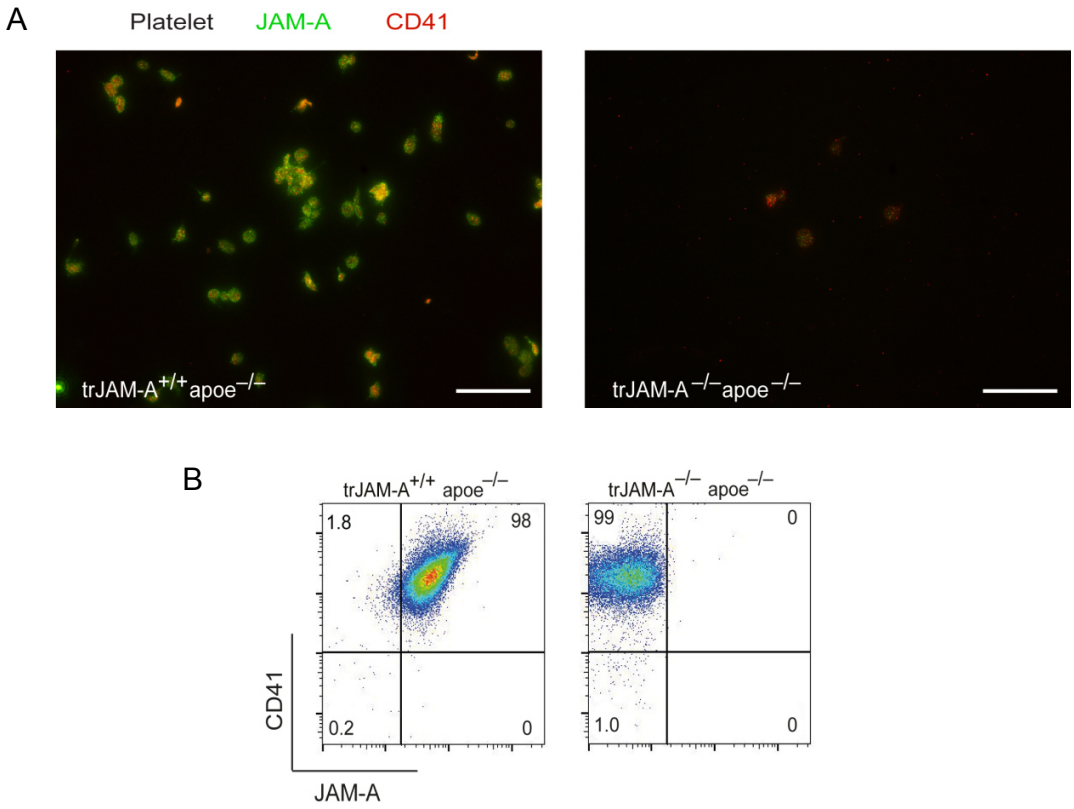


Figure 5: JAM-A deletion on platelets. JAM-A expression on isolated platelets from trJAM-A$^{+/+}$apoe$^{-/-}$ and trJAM-A$^{-/-}$apoe$^{-/-}$ mice were detected by immunofluorescence staining (JAM-A: green, CD41: red) and flow cytometry (B). Scale bar=20µm (A). Representative pictures of more than 3 independent experiments.

4.1.2 JAM-A expression in arterial wall

To localize JAM-A expression in vessel wall, we did immunofluorescence staining in carotid arteries from trJAM-A$^{+/+}$apoe$^{-/-}$ and trJAM-A$^{-/-}$apoe$^{-/-}$mice. vWF as a marker of ECs was co-localized in intima with JAM-A (Figure 6A). In arterial media, JAM-A signal was detectable in SMCs and merged with α-SMA signal (Figure 6B). To quantify JAM-A expression in arterial wall, western blot showed JAM-A bands from homogenates of aortae or carotid arteries at same level of expression in both groups (data shown in our paper). This means that specific genetic deletion of platelet JAM-A did not affect JAM-A expression in arterial wall.

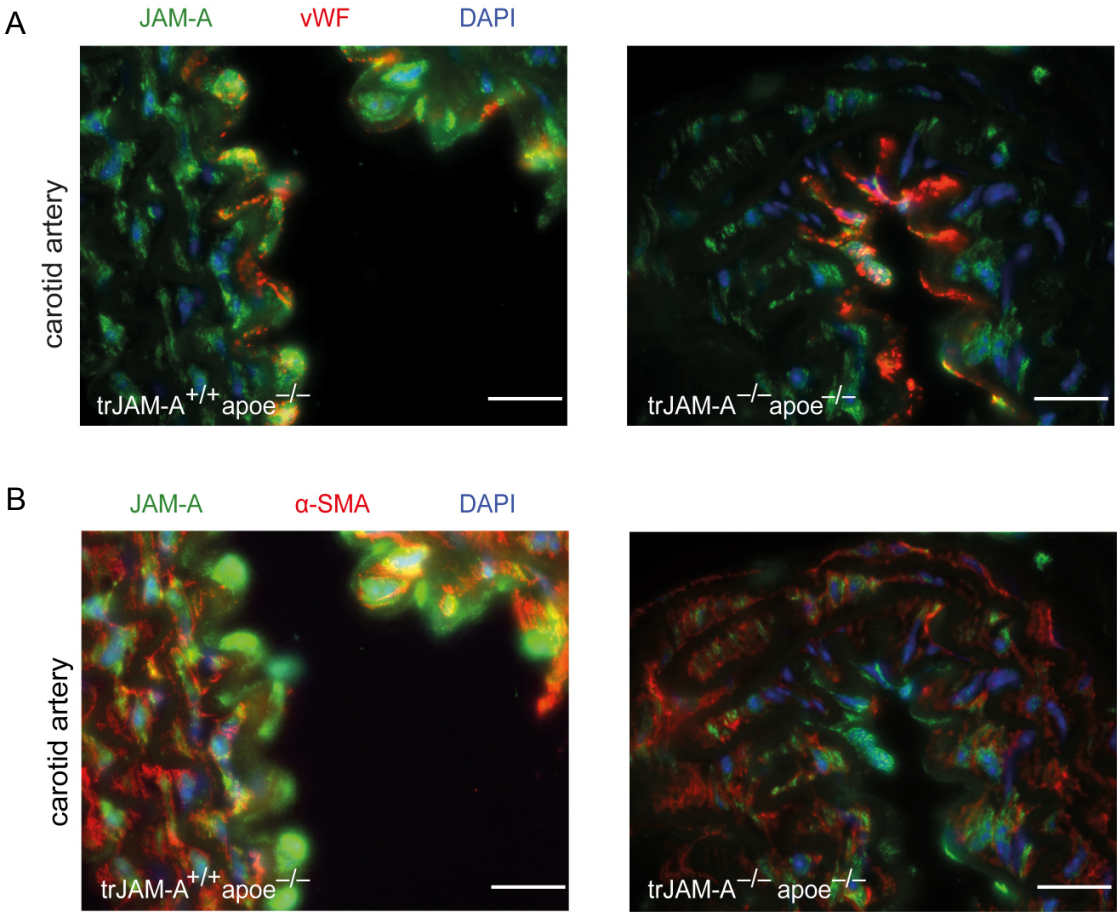


Figure 6: JAM-A expression in arterial wall. Immunofluorescence images of carotid arteries from trJAM-A$^{+/+}$apoe$^{-/-}$ and trJAM-A$^{-/-}$apoe$^{-/-}$ mice. ECs (A, overlays, JAM-A: green, vWF: red and nuclei: blue) and SMCs (B, overlays, JAM-A: green, SMCs: red and nuclei: blue). Scale bar=20 µm. Independent experiments were repeated more than 3 times.

4.1.3 Related adhesion molecules expression on platelets

To search the effects of trJAM-A deletion on the expression of related adhesion molecules, JAM-C and ESAM were stained by fluorescence antibodies and the expression levels on platelets were depicted as mean fluorescence intensity by flow cytometry. JAM-C was detected on isolated platelets from trJAM-A$^{+/+}$apoe$^{-/-}$ and trJAM-A$^{-/-}$apoe$^{-/-}$ mice and quantification of JAM-C expression on platelets by mean fluorescence intensity showed no significant difference between trJAM-A$^{+/+}$apoe$^{-/-}$ and

trJAM-A$^{-/-}$apoe$^{-/-}$ mice (Figure 7A). Although ESAM expression on platelets was showed a slight decrease trend in trJAM-A$^{-/-}$apoe$^{-/-}$ mice compared with trJAM-A$^{+/+}$ apoe$^{-/-}$ mice, but there was no significant difference (Figure 7B). Hence, the absence of trJAM-A did not affect related adhesion molecules expression on platelets.

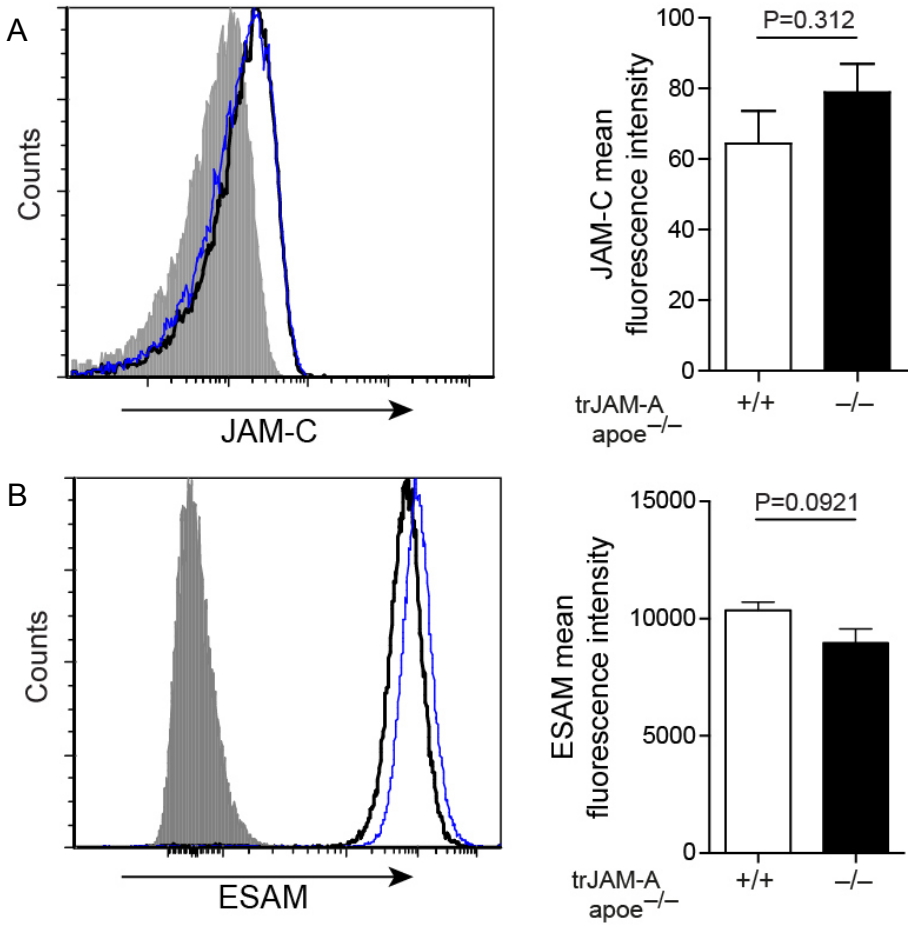


Figure 7: JAM-C and ESAM expression on platelets. JAM-C (A) and ESAM (B) were stained by fluorescence antibodies on isolated platelets from trJAM-A$^{+/+}$apoe$^{-/-}$ (blue curve) and trJAM-A$^{-/-}$apoe$^{-/-}$ mice (black curve), compared with controls (grey curve) and their fluorescence intensity was measured by flow cytometry. N=3 mice per group. Fluorescence intensity was quantified and represented as mean±SEM, P values were calculated by Student's t-test.

4.2 Platelets and white blood cells counts in mouse blood

To determine effect of trJAM-A deficiency on blood cell populations, blood in EDTA-anti-coagulated tubes was isolated from trJAM-$A^{+/+}$ $apoe^{-/-}$ and trJAM-$A^{-/-}$ $apoe^{-/-}$ mice fed HFD for 0, 2, 6, 12 weeks. According to table1, platelet number in blood displayed no significant difference between trJAM-$A^{+/+}apoe^{-/-}$ and trJAM-$A^{-/-}apoe^{-/-}$ mice ($P<0.05$), but platelet count increased with the course of HFD.

Mean platelet volume (MPV) as a measurement of average platelet size in blood indicates platelet production in bone marrow. Once MPV is higher, it means the body is producing more platelets. Table 1 showed that MVP was in normal range (4.6-7.3 femtoliter) and there was no significant difference between trJAM-$A^{+/+}apoe^{-/-}$ and trJAM-$A^{-/-}apoe^{-/-}$ mice ($P>0.05$) at different time points of HFD.

The number of white blood cells showed stable and little variation between trJAM-$A^{+/+}$ $apoe^{-/-}$ and trJAM-$A^{-/-}apoe^{-/-}$ mice ($P>0.05$) fed HFD for 0, 2, 6 or 12 weeks. Among them, the populations of lymphocytes, monocytes and neutrophils displayed no significant difference after platelet JAM-A deletion ($P>0.05$). Hence, trJAM-A deficiency did not alter the numbers of white blood cells in blood.

genotype	platelets	MPV	WBC	lymphocytes	monocytes	neutrophils
trJAM-$A^{+/+}$, w/o HFD	386±148	6.57±0.35	5.08±0.85	5.25±0.60	0.225±0.03	1.63±0.23
trJAM-$A^{-/-}$, w/o HFD	499±157	6.00±0.17	5.50±0.15	5.73±0.07	0.303±0.03	1.47±0.15
p-value	0.63	0.23	0.22	0.38	0.09	0.62
trJAM-$A^{+/+}$, 2w HFD	719±36.9	6.00±0.05	6.23±0.48	4.56±0.39	0.231±0.03	1.44±0.09
trJAM-$A^{-/-}$, 2w HFD	766±64.9	5.97±0.06	6.18±0.38	4.47±0.25	0.241±0.03	1.48±0.19
p-value	0.56	0.66	0.94	0.83	0.81	0.40
trJAM-$A^{+/+}$, 6w HFD	765±130	6.67±0.31	4.75±0.67	3.18±0.25	0.167±0.07	1.40±0.43
trJAM-$A^{-/-}$, 6w HFD	839±125	6.66±0.41	5.24±0.38	3.66±0.26	0.211±0.03	1.38±0.14
p-value	0.78	0.71	0.24	0.26	0.21	0.26
trJAM-$A^{+/+}$,12w HFD	947±124	6.76±0.17	4.84±0.47	3.03±0.33	0.193±0.03	1.62±0.02
trJAM-$A^{-/-}$,12w HFD	1286±146	6.45 ± 0.13	4.40 ± 0.46	2.77 ± 0.28	0.164 ± 0.03	1.46 ± 0.17
p-value	0.09	0.18	0.37	0.58	0.47	0.57

Table 1: Platelet, mean platelet volume (MPV) and white blood cell counts (WBC). The whole blood was isolated into EDTA tubers from trJAM-$A^{-/-}apoe^{-/-}$ and trJAM-$A^{+/+}apoe^{-/-}$ mice fed without (w/o) HFD and with HFD for 2 weeks (2w), 6 weeks (6w), 12 weeks (12 w). Values are shown as (mean ±SEM)*10^9 cells/L blood, except MPV as

(mean ±SEM)*femtoliter. N=7-14 mice per group and all P values were calculated by Student's t-test.

4.3 Lipid metabolism after platelet–JAM-A deletion

All mice were used in this project with apoe gene deficiency background, which caused hypercholesterolemia as the main driving force of lesion growth. Lipid metabolism indicated by plasma levels of cholesterol and triglycerides was measured. The data showed that there was no significant difference (P>0.05) between trJAM-$A^{-/-}$$apoe^{-/-}$ and trJAM-$A^{+/+}$$apoe^{-/-}$ mice fed HFD for 2 weeks or longer.

genotype	**cholesterol (mg/dl)**	**triglycerides (mg/dl)**
trJAM-$A^{+/+}$$apoe^{-/-}$, 2w HFD	984 ± 132	164 ± 40.9
trJAM-$A^{+/+}$$apoe^{-/-}$, 2w HFD	1105 ± 125	141 ± 20.5
p-value	0.51	0.62
trJAM-$A^{+/+}$$apoe^{-/-}$, 6w HFD	918 ± 132	165 ± 31.5
trJAM-$A^{+/+}$$apoe^{-/-}$, 6w HFD	951 ± 161	184 ± 48.8
p-value	0.88	0.96
trJAM-$A^{+/+}$$apoe^{-/-}$,12w HFD	1222 ± 121	178 ± 26.5
trJAM-$A^{+/+}$$apoe^{-/-}$,12w HFD	1349 ± 146	222 ± 30.5
p-value	0.51	0.29

Table 2: Plasma cholesterol and triglyceride levels. Plasma was isolated from trJAM-$A^{-/-}$$apoe^{-/-}$ and trJAM-$A^{+/+}$$apoe^{-/-}$ mice fed HFD for 2 weeks (2w), 6 weeks (6w) and 12 weeks (12 w). The levels of cholesterol and triglyceride in plasma are expressed as (mean ±SEM)*mg/dl. N=7-13 mice per group and all P values were calculated by Student's t-test.

4.4 JAM-A deficiency results in platelet hyperreactivity

4.4.1 JAM-A deficiency increases platelet aggregation

To assess platelet aggregation upon activation, multiplate was used to measure platelets aggregation under agonist stimulation. Whole blood was isolated retro-orbitally from trJAM-$A^{+/+}$ $apoe^{-/-}$ and trJAM-$A^{-/-}apoe^{-/-}$ mice into citrate-coated tubes. After dilution, blood was added agonist, such as thrombin and ADP, and measured under multiplate. 2 curves per sample (as internal control) depicted platelet aggregation progress and area under the curves as aggregation degree (Figure 8A, B). Under lower concentrations of thrombin, from 0.02 U/ml to 0.5 U/ml, JAM-$A^{-/-}apoe^{-/-}$ platelets aggregated faster and the degree showed significantly elevated, compared with JAM-$A^{+/+}apoe^{-/-}$platelets, but there was no significant difference under 1U/ml thrombin (Figure 8C). Similar results were displayed under ADP stimulation (Figure 8D). The enhanced aggregation to different agonists revealed JAM-$A^{-/-}apoe^{-/-}$platelet hyperreaction, which was consistent with previous findings[181, 182]. These results suggest that JAM-A acts an endogenous inhibitor of platelet function.

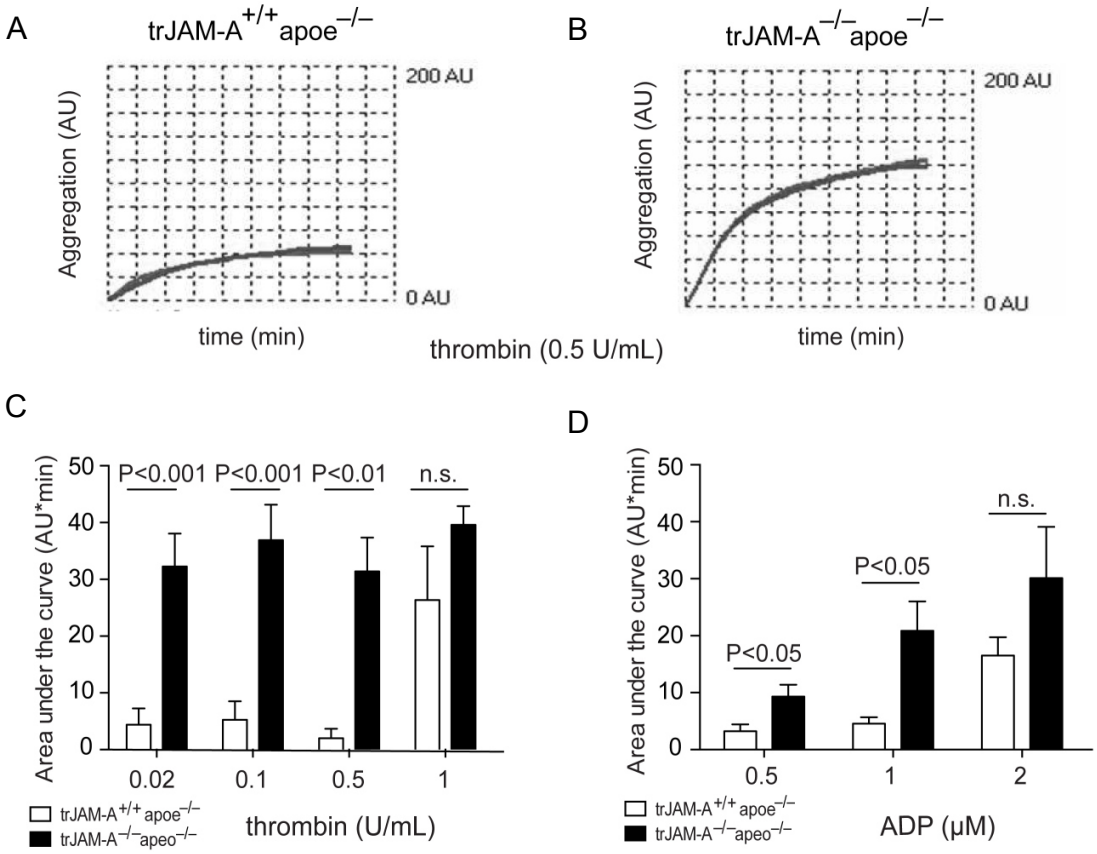


Figure 8: Platelet hyperreactivity in trJAM-A$^{-/-}$apoe$^{-/-}$ mice. Representative images of thrombin activation (0.5 U/mL) to JAM$^{+/+}$apoe$^{-/-}$ (A) and JAM-A$^{-/-}$apoe$^{-/-}$ platelets (B). Platelet aggregation due to thrombin (C) and ADP (D) activation is presented as area under the curve in aggregation units (AU) in 5 minutes, expressed as (mean ±SEM) AU*min. N=8-15 mice per group. P<0.05 showed significant difference and all P values were calculated by ANOVA with Tukey's post test.

4.4.2 JAM-A negatively regulates platelet activity through Integrin αIIbβ3

Previous observations have shown that platelet aggregation is mediated by integrin αIIbβ3, which bind with vWF or fibrinogen triggering outside-in signaling. To address whether the hyperaggregation of JAM-A$^{-/-}$apoe$^{-/-}$ platelets was also mediated by integrin αIIbβ3, its specific antagonist tirofiban was used. After tirofiban inhibition, the aggregation of JAM-A$^{-/-}$apoe$^{-/-}$ platelets was suppressed under 0.5 U/mL thrombin stimulation (Figure 9A, B) and quantification of areas under the curves showed significantly decrease (P<0.05, between 2 black bars in Figure 9C). Notably, the

enhanced aggregation of JAM-A$^{-/-}$apoe$^{-/-}$ platelets (P<0.01, Figure 9C left) was normalized by tirofiban to the level of JAM-A$^{+/+}$apoe$^{-/-}$ platelets, as there was no significant difference between JAM-A$^{+/+}$apoe$^{-/-}$ platelets and JAM-A$^{-/-}$apoe$^{-/-}$ platelets (P>0.05, Figure 10C right). To further validate this observation, ADP stimulated platelets also showed similar results (Figure 10D). Tirofiban dramatically suppressed JAM-A$^{-/-}$ apoe$^{-/-}$ platelet aggregation under ADP activation (Black bars) and showed no significant difference with JAM-A$^{+/+}$apoe$^{-/-}$ platelets (P>0.05, Figure 10D right). These data indicate that integrin αIIbβ3 mediates hyperreaggregation of JAM-A$^{-/-}$ apoe$^{-/-}$ platelets.

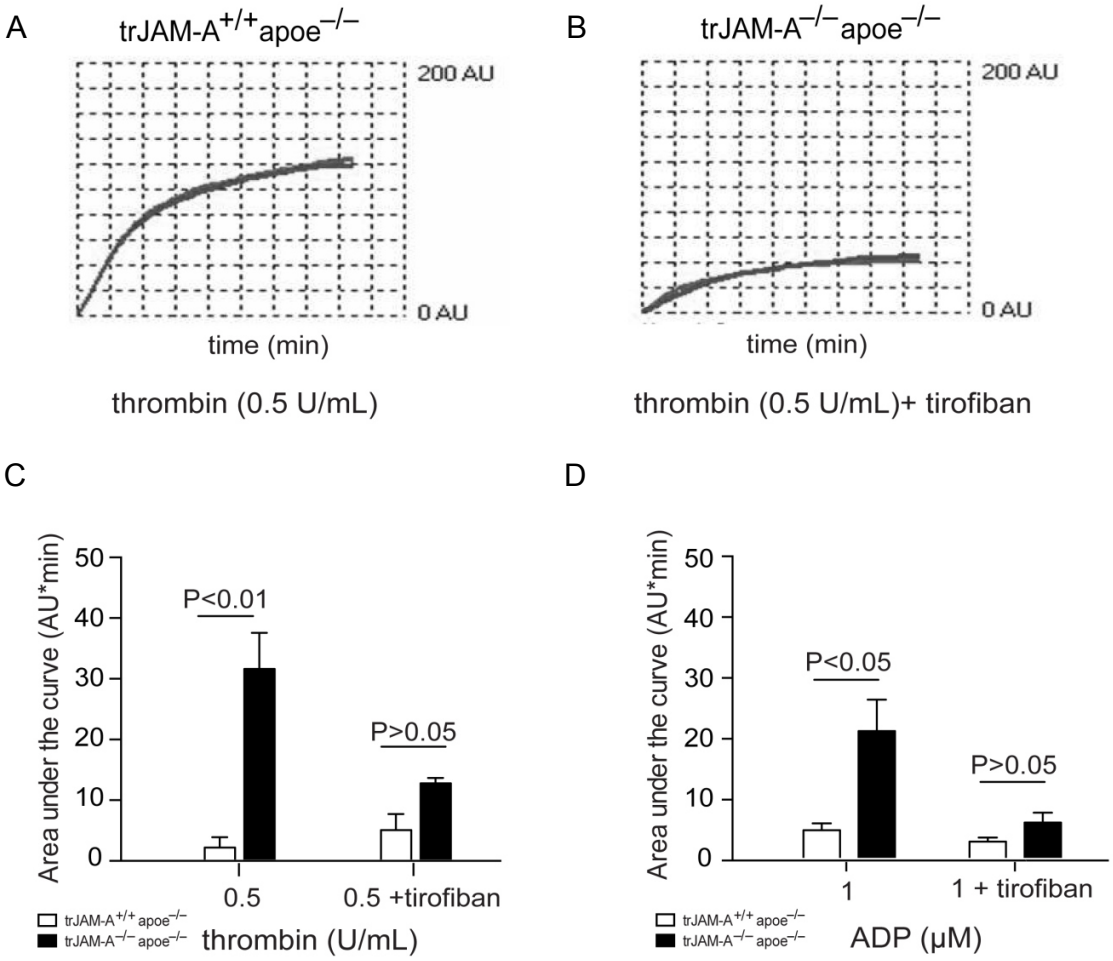


Figure 9: Integrin αIIbβ3 controls platelet activity. Representative images of thrombin (0.5 U/mL) activation (A) and tirofiban (1 μg/mL) inhibition (B) to JAM-A$^{-/-}$ apoe$^{-/-}$ platelets are shown. Curves in the images recorded the process of platelet aggregation. Aggregation of JAM-A$^{+/+}$apoe$^{-/-}$ and JAM-A$^{-/-}$apoe$^{-/-}$ platelets activated by

thrombin(C) or ADP (D) in absence or presence of tirofiban is presented as area under the curve in aggregation units (AU), expressed as (mean ±SEM) AU*min. N=8-15 mice per group and all P values were calculated by ANOVA with Tukey's post test.

4.4.3 JAM-A inhibits c-Src activity in integrin αIIbβ3 outside-in signaling

After platelet activation, integrin αIIbβ3 conformation converted from its low-affinity state to a high-affinity state is referred as inside-out signaling[173]. Ligand binding to the activated integrin triggers cascade signaling events to stabilize platelet aggregates, named outside-in signaling[174]. To investigate the downstream molecule cascade of integrin αIIbβ3 outside-in signaling, the role of Src kinase in platelet aggregation was assessed by multiplate. PP2 as an inhibitor of Src significantly blocked the increased aggregation of JAM-A$^{-/-}$apoe$^{-/-}$ platelets, compared with JAM-A$^{+/+}$apoe$^{-/-}$ platelets stimulated by ADP (Figure 10A) or thrombin(Figure 10B). DMSO, as solvent of PP2, was used additionally as control. JAM-A$^{-/-}$apoe$^{-/-}$ platelets still showed significant hyperreaggregation compared with JAM-A$^{+/+}$apoe$^{-/-}$ platelets (Figure 10A, B), which indicated that DMSO did not affect platelet function. These data revealed that Src combined with integrin αIIbβ3, confirmed by previous observations[181, 182], regulates platelet aggregation. Taken together, Src might regulate platelets aggregation in signaling downstream of integrin.

Src activity was further explored, which is reflected by Src phosphorylation state at residue Y418. Under non-activated conditions, the state of Src Y418 phosphorylation in isolated JAM-A$^{-/-}$apoe$^{-/-}$ platelets showed no significant difference with JAM-A$^{+/+}$apoe$^{-/-}$ platelets. Additionally, the quantification of fluorescence intensity of Src Y418 showed that JAM-A deficiency did not affect Src Y418 phosphorylation after thrombin stimulation (Figure 10C, D), as thrombin activates platelets leading integrin to high affinity state through inside-out signaling. The statistic comparison showed no difference ($P>0.05$, Figure 10C, D) suggested that Src activity and JAM-A was not involved in integrin αIIbβ3 inside-out signaling.

As the ligand of integrin αIIbβ3, Fg can trigger integrin outside-in signaling and activate platelets. Isolated platelets adhered to BSA- (as control) or Fg-coated dishes and then Src Y418 phosphorylation in platelet activation was measured in flow cytometry. In BSA

group, there was no significant difference of Src Y418 phosphorylation in platelets with or without JAM-A expression. In addition, with and without Fg stimulation, JAM-$A^{+/+}$apeo$^{-/-}$ platelets showed no significant difference of Src Y418 phosphorylation (Figure 10F, 2 white bars). In contrast, without JAM-A expression, Fg stimulated platelets showed higher Src Y418 phosphorylation, as the fluorescence intensity in JAM-$A^{-/-}$apoe$^{-/-}$ platelets displayed stronger (Figure 10E), and the data showed significantly increased in trJAM-$A^{-/-}$apoe$^{-/-}$ mice ($P<0.001$, Figure 10F). It illustrates that Src activity was increased by JAM-A deficiency during integrin ligating to Fg. In another words, JAM-A might decrease the thresholds of Src activation in integrin outside-in signaling.

Taken together, JAM-A deficiency led to platelet hyperreactivity due to elevated Src activity in integrin αIIbβ3 outside-in signaling.

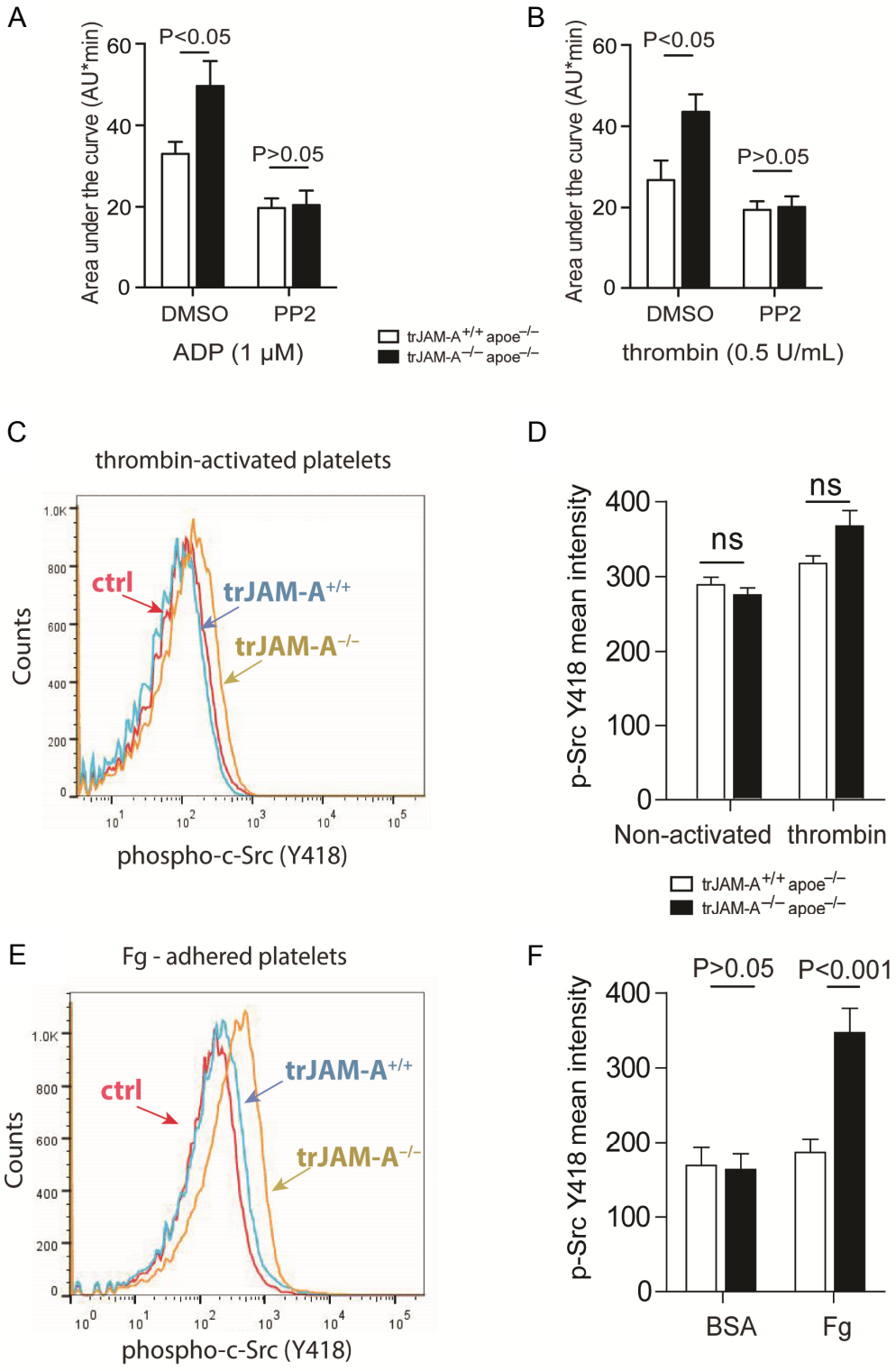


Figure 10: JAM-A suppressed c-Src activity in integrin αIIbβ3 outside-in signaling. Aggregation of JAM-A$^{+/+}$apoe$^{-/-}$ (white bars) and JAM-A$^{-/-}$apoe$^{-/-}$ (black bars) platelets

treated with DMSO (as control) or Src inhibitor PP2 (20μM) after activation by 1 μM ADP (A) or 0.5 U/ml thrombin (B). Representative flow cytometry histogram (C) of phosphorylated c-Src Y418 residue staining in thrombin-activated platelets (red: control, blue: trJAM-$A^{+/+}$$apoe^{-/-}$, orange: trJAM-$A^{-/-}$$apoe^{-/-}$) and quantification of phosphorylated c-Src mean intensity in non-activated and thrombin-activated platelets was shown (D). Representative flow cytometry histogram (E) of phosphorylated c-Src Y418 residue staining in adherent platelets to Fg (red: control, blue: trJAM-$A^{+/+}$$apoe^{-/-}$, orange: trJAM-$A^{-/-}$$apoe^{-/-}$) and quantification of phopho-c-Src mean intensity in platelets adhered to BSA or Fg was shown (F). The data represent mean ± SEM. N=4-7 mice per group and all P values were calculated by ANOVA with Tukey's post test.

4.4.4 PTP1 catalyzes JAM-A de-phosphorylation after platelet activation

In resting platelets, tyrosine phosphorylated JAM-A (p-tyr/JAM-A) maintains c-Src inactivity by recruitment of c-Src-inhibiting kinase CSK to integrin αIIbβ3, to form JAM-A/CSK/c-Src/integrin αIIbβ3 complex. Upon ligation of integrin αIIbβ3, JAM-A is dephosphorylated followed by CSK dissociation from the complex and loses the maintenance of Src inactivity[2]. How JAM-A is dephosphorylated remains unclear, so we performed immunoprecipitation to investigate the related enzymes using human and mouse platelets.

Protein tyrosine phosphates (PTPs) are responsible for tyrosine de-phosphorylation. Among 20 PTPs in platelet signaling events, PTPN1 (PTP1B) was shown to associate with integrin complex and regulates platelet outside-in signaling[187]. In human platelet lysates, JAM-A and PTPN1 were detectable, but the antibody against phosphotyrosine was not able to detect the p-tyr/JAM-A in western blot (data shown in our paper). Immunoprecipitation revealed that p-tyr/JAM-A was co-precipitated with JAM-A detectable in resting and activated platelets, but it decreased in Fg-activated platelets, which was inhibited by broad-spectrum PTP inhibitor IV (Figure 11 A). This suggested that JAM-A de-phosphorylation was catalyzed by PTPs after integrin activation.

To confirm our observation, we performed JAM-A immunoprecipitation using mouse platelets under specific PTPs inhibitors (Figure 11B). p-tyr/JAM-A in Fg activated

platelets showed obviously decrease versus in resting platelets, which can be reversed by broad-spectrum PTP inhibitor XXXI, PTP1 specific inhibitor or 10 µM NSC-87877. Interestingly, NSC-87877, an inhibitor of PTPN6 and PTPN11 (SHP1 and SHP2) at lower concentration (0.7 µM), did not affect JAM-A de-phosphorylation. At higher concentration (10 µM) of NSC-87877 also blocks PTPN1 suppression of JAM-A de-phosphorylation (Figure 11B). Taken together, PTPN1 might be responsible PTP that catalyzes JAM-A de-phosphorylation during Fg-triggered integrin outside-in signaling. Other PTPs might be involved in JAM-A de-phosphorylation to a lesser extent.

Upon these results, JAM-A as a member of CSK/c-Src/integrin αIIbβ3 complex negatively regulates integrin αIIbβ3 outside-in signaling. In other words, JAM-A deficiency results in platelet hyperreactivity through decreased threshold of c-Src activity in platelet outside-in signaling. After integrin activation, JAM-A de-phosphorylation is catalyzed by PTPN1.

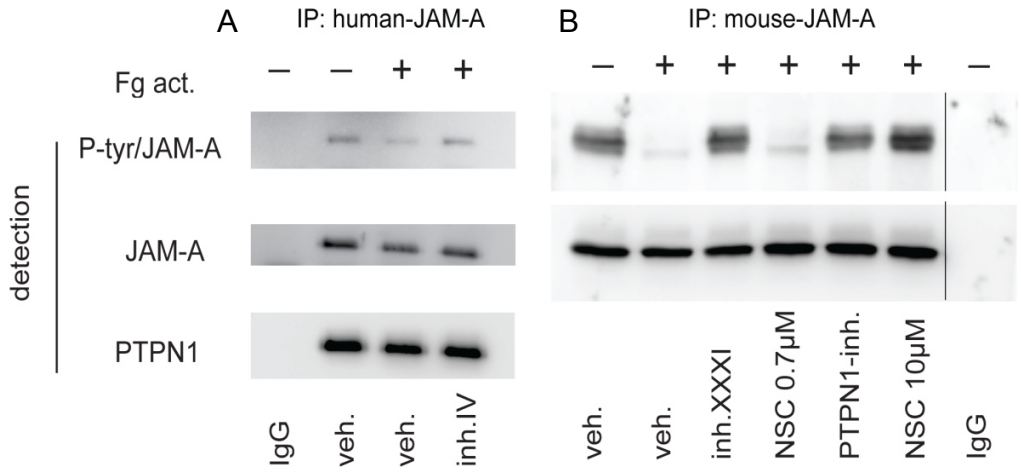


Figure 11: JAM-A de-phosphorylation by PTPN1 in integrin outside-in signaling. Platelets were isolated from human (A) or mouse (B) blood. Representative immunoblots of immunoprecipitated JAM-A were from adherent platelets on BSA- or fibrinogen (Fg)-coating dishes with PTP inhibitor or vehicle (veh). Antibodies against to phosphotyrosine, JAM-A or PTPN1 were used. IgG was used as control. Broad-spectrum PTP inhibitor IV (inh.IV) and XXXI (inh. XXXI). NSC-87877 (NSC 0.7 µM) as an inhibitor of PTPN6 and PTPN11. NSC at concentration of 10 µM blocks PTPN1. PTPN1 specific inhibitor was shown as PTPN1-inh. This experiment was repeated 3 times.

4.5 Platelet–JAM-A deficiency enhances vascular inflammation

4.5.1 Augmented adhesion of JAM-A–deficient platelets to atherogenic vessel wall

To study platelet interaction with atherogenic wall under JAM-A regulation, left carotid arteries were explanted from trJAM-$A^{+/+}$$apoe^{-/-}$ and trJAM-$A^{-/-}$$apoe^{-/-}$ mice fed a HFD for 2 weeks and mounted in a chamber for TPLSM ex vivo. After whole blood incubation with anti-CD41-FITC antibody and vessel wall labeled with anti-CD31-eFluor450, blood was perfused across the lumen of carotid artery (blood and carotid artery from the same mouse). TPLSM images demonstrated that endothelium (blue) was intact and not damaged by handling. Notably, platelets adhered to endothelium and more JAM-$A^{-/-}$ $apoe^{-/-}$ platelets adhered to carotid arterial wall, compared to JAM-$A^{+/+}$$apoe^{-/-}$ platelets, according to the absolute JAM-$A^{-/-}$$apoe^{-/-}$ platelet number per field (in certain area). This indicates that JAM-A negatively regulates platelet interaction with atherogenic endothelial cells (Figure 12A, B).

To further confirm that JAM-A regulates platelet interaction with atherogenic vessel wall, tirofiban, as the specific inhibitor of integrin αIIbβ3 was applied in the perfused blood. Tirofiban diminished JAM-$A^{-/-}$$apoe^{-/-}$ platelet adhesion on ECs (Figure 12C). The quantification showed that JAM-$A^{-/-}$$apoe^{-/-}$ platelets on ECs significantly decreased after tirofiban inhibition, compared to JAM-$A^{+/+}$$apoe^{-/-}$ platelets. There is no significant difference of adhesion between JAM-$A^{+/+}$$apoe^{-/-}$ and JAM-$A^{-/-}$$apoe^{-/-}$ platelets inhibited by tirofiban, suggesting that tirofiban can inhibit the hyperreactivity of JAM-$A^{-/-}$ $apoe^{-/-}$ platelets through integrin αIIbβ3 (Figure 12D).

Upon vitro, ex vivo or vivo experiments, platelet and leukocyte were co-localized on inflammatory endothelium and atherogenic vessel wall. Additionally, platelet hyperreactivity due to JAM-A deficiency increased platelets and leukocytes adhesion to endothelium, indicating increased leukocytes recruitment to atherogenic sites (*data are published in Karshovska E, Zhao Z et al, Circ Res. 2015 Feb. 13; 116(4):587-99*).

Taken together, JAM-A deficiency caused platelet hyperreactivity and increased platelet adhesion to atherosclerosis-prone endothelium through integrin αIIbβ3.

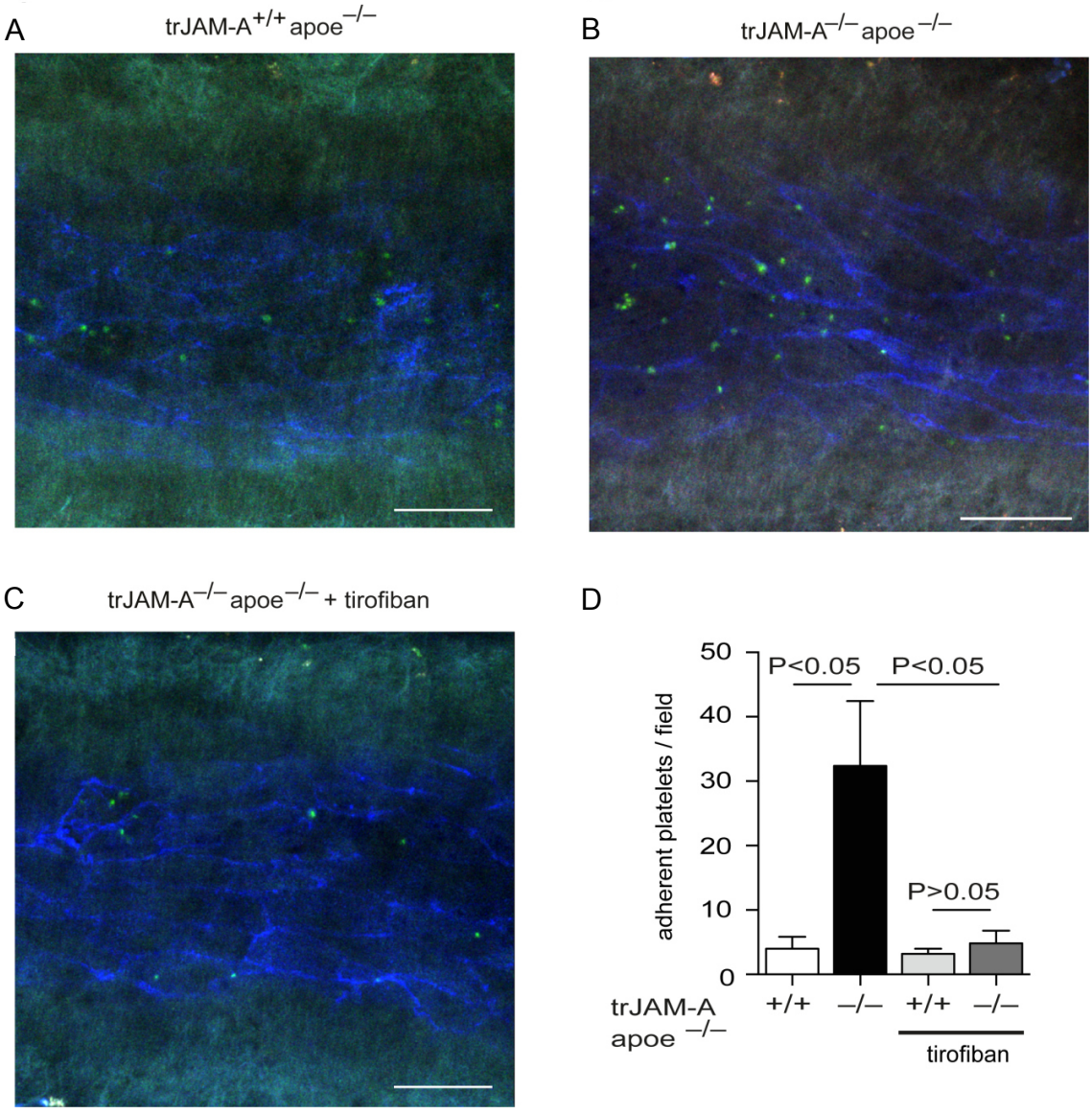


Figure 12: Increased adhesion of JAM-A$^{-/-}$apoe$^{-/-}$ platelets to atherogenic endothelium. TPLSM images of adherent platelets (CD41, green) on endothelium (CD31, blue) in carotid arteries ex vivo from trJAM-A$^{+/+}$apoe$^{-/-}$ and trJAM-A$^{-/-}$apoe$^{-/-}$ mice fed a HFD for 2 weeks. Isolated left carotid artery labeled with anti-CD31 eFluor®450-conjugated antibody (3.5µg/ml, eBioscience) from the mouse was mounted in a chamber and perfused by its whole blood incubated with anti-CD41 FITC-conjugated antibody

(3.7µg/ml, eBioscience) for 30 minutes without (A, B) or with tirofiban (1µg/mL, C). Blood was perfused along the mounted and pressurized vessel for 10 minutes at 0.5ml/min. Background color was from autofluorescence in vessel wall. Adherent platelet number per visual field was counted. Each carotid artery was imaged more than 6 fields (D). Scale bar=20µm (A, B, C). N=5-8 mice per group and P values were calculated by Kruskal-Wallis test with Dunn´s post test.

4.5.2 Increased plasma levels of chemokines due to JAM-A–deficient platelets

As previous studies, platelets store and release many inflammatory proteins, including various kinds of chemokines, such as platelet factor 4 (PF4, CXCL4) and regulated on activation, normal T cells expressed and secreted (RANTES, CCL5), which can increase recruitment of mononuclear cells to the inflamed vessel wall[82, 189]. Thus, CXCL4 and CCL5 were chosen out as the investigated targets in atherosclerosis. Immunofluorescence staining showed that CXCL4 was rich on the surface and in cytoplasm of platelets and its expression level was higher than CCL5 in JAM-A wild type mice (Figure 13A, C). Interestingly, CXCL4 and CCL5 in JAM-A$^{-/-}$ platelets diminished, as fluorescence intensity was weaker in JAM-A$^{-/-}$ apoe$^{-/-}$ platelets compared to JAM-A$^{+/+}$apoe$^{-/-}$ platelets (Figure 13A, C), indicating that JAM-A deficiency elevated chemokines release from platelets.

Notably, ELISA data showed that CXCL4 concentration in plasma increased in trJAM-A$^{-/-}$apoe$^{-/-}$ mice compared to trJAM-A$^{+/+}$apoe$^{-/-}$ mice, significantly in advanced stage of atherosclerosis (HFD 12 weeks). As the disease progression, the plasma level of CXCL4 increased from 2 weeks to 12 weeks HFD in trJAM-A$^{-/-}$apoe$^{-/-}$ mice, while it was stable in trJAM-A$^{+/+}$apoe$^{-/-}$ mice (Figure 13B).

Plasma CCL5 concentration was markedly raised in trJAM-A$^{-/-}$apoe$^{-/-}$ mice verses in trJAM-A$^{+/+}$apoe$^{-/-}$ mice during earlier stages of atherosclerosis (HFD 2 weeks, 6weeks), although there was no significant difference at late stage (Figure 13D), indicating that trJAM-A deficiency might augment CCL5 secretion from platelets. However, the baseline levels of CCL5 and CXCL4 in plasma without HFD showed no significant

difference between trJAM-A$^{-/-}$apoe$^{-/-}$ and trJAM-A$^{+/+}$apoe$^{-/-}$ mice (data no shown), suggesting that the increased CCL5 and CXCL4 in plasma might require HFD co-stimulation with JAM-A deficiency on platelets.

These data revealed that trJAM-A deficiency under hyperlipidemia led to intracellular CXCL4 and CCL5 release in platelets paralleled with higher levels in plasma during the context of atherosclerosis. Taken together, it implied that JAM-A deficiency might augment chemokines (CXCL4 and CCL5) secretion from platelets into plasma. In turn, increased chemokines might recruit more leukocytes to atherosclerosis-prone sites.

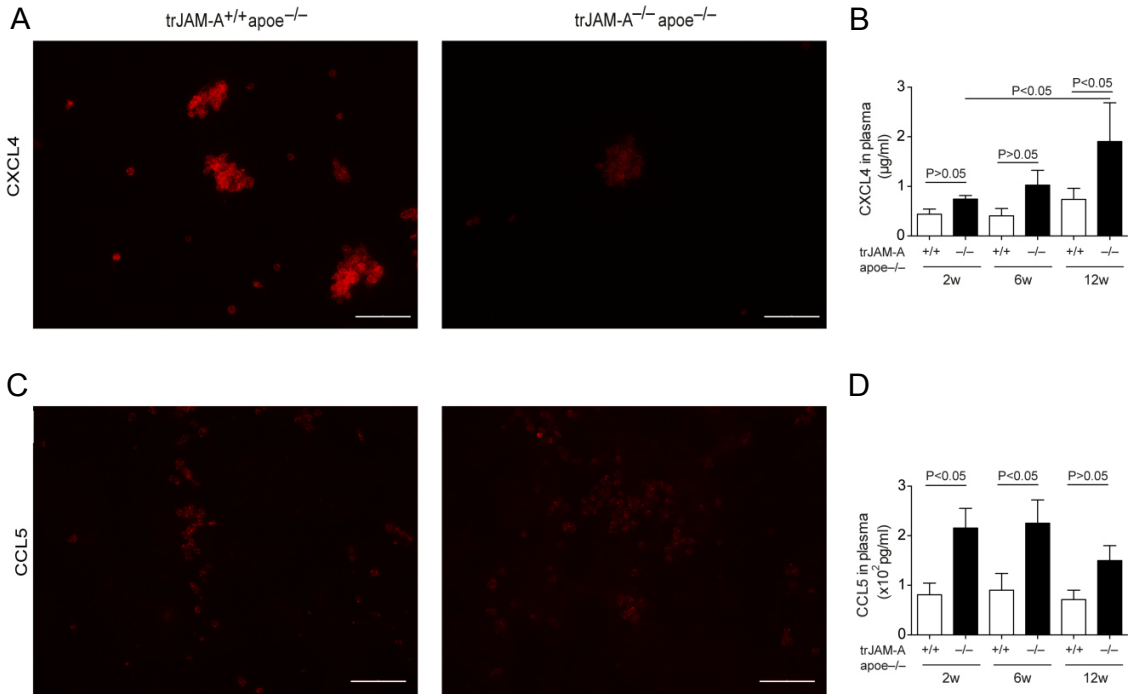


Figure 13: Role of platelet–JAM-A in Chemokines release. Representative images of CXCL4 in platelets from trJAM$^{+/+}$apoe$^{-/-}$ and trJAM$^{-/-}$apoe$^{-/-}$ mice fed 12 weeks (A). CCL5 staining in platelets from trJAM$^{+/+}$apoe$^{-/-}$ and trJAM$^{-/-}$apoe$^{-/-}$ mice fed 2 weeks (C). Scar bar: 20µm. Each experiment was repeated more than 3 times. The levels of CXCL4 and CCL5 in platelet poor plasma isolated from trJAM$^{+/+}$apoe$^{-/-}$ and trJAM$^{-/-}$apoe$^{-/-}$ mice fed 2 weeks, 6 weeks or 12 weeks (B, D). CXCL4 concentration in plasma represents (mean±SEM) µg/mL and CCL5 concentration in plasma represents (mean±SEM)*10^2 pg/mL. N=7-14 mice per group. All P values were calculated by 1-way ANOVA with Bonferroni´s post test.

4.5.3 Enhanced interactions of JAM-A–deficient platelets with leukocytes

Previous studies have found that activated platelets can form complexes with leukocytes in cardiovascular disease[105, 190, 191]. To address whether JAM-A$^{-/-}$apoe$^{-/-}$ platelets hyperreactivity contributes to the interactions with leukocytes, isolated platelets were activated by thrombin and then incubated with leukocytes. Flow cytometry measured the percentage of complexes, platelet-monocyte (Figure 14A) and platelet-neutrophil (Figure 14B) in total leukocytes and found that JAM-A$^{-/-}$apoe$^{-/-}$ platelets combined to leukocytes more than JAM-A$^{+/+}$apoe$^{-/-}$ platelets. The statistic results showed that complex percentage of JAM-A$^{-/-}$apoe$^{-/-}$ platelets with monocytes was significantly elevated, compared to JAM-A$^{+/+}$apoe$^{-/-}$ platelet-monocyte complexes. To further explore the molecular mechanisms, tirofiban blocking integrin αIIbβ3 diminished the enhanced interactions between JAM-A$^{-/-}$apoe$^{-/-}$ platelets and monocytes (Figure 14A, C). The similar results of platelet-neutrophil complex formation were also found (Figure 14B, D). These data indicates that trJAM-A deficiency can promote platelet-leukocyte complex formation, which can facilitate leukocyte tethering on endothelial cells[190, 191]. It implies that platelet hyperreactivity due to JAM-A deficiency might increase leukocyte recruitment from blood to the atherogenic sites through platelet-leukocyte complex formation in blood circulation.

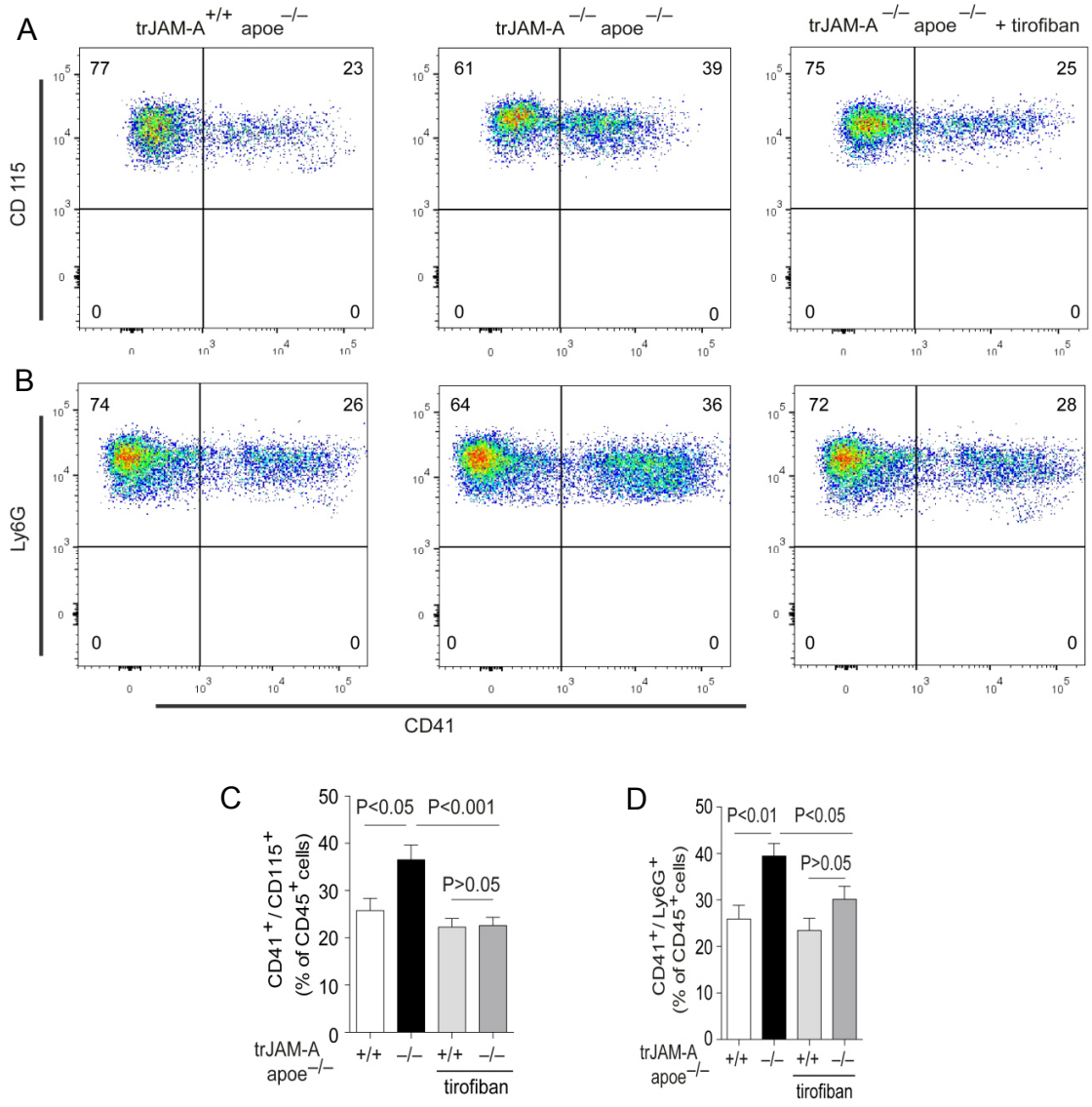


Figure 14: platelet–JAM-A deficiency enhanced platelet-leukocyte complex formation. Representative flow cytometry histograms of platelet ($CD41^+$)-monocyte ($CD115^+$) interactions (A) and platelet-neutrophil ($ly6G^+$) complexes (B) in absence or presence of tirofiban (1 µg/ml). Platelets were activated by thrombin (0.5U/ml) and then incubated with isolated leukocytes ($CD45^+$). Quantification of complexes percentage was shown (C, D). Data: mean±SEM (n=7-14) and all P values were calculated by 1-way ANOVA with Bonferroni´s post test.

4.6 Platelet–JAM-A deficiency accelerates atherosclerosis

To investigate the gain-of-function of JAM-A–deficient platelets during atherosclerotic progress, the plaques were assessed in trJAM-A$^{-/-}$apoe$^{-/-}$ and trJAM-A$^{+/+}$apoe$^{-/-}$ mice fed HFD for 2 weeks, 6 weeks or 12 weeks.

4.6.1 JAM-A–deficient platelets propagate aortic atherosclerosis

To clarify the effect of trJAM-A deficiency in atherosclerosis, aortic plaques were measured to assess atherosclerotic burden. trJAM-A$^{-/-}$apoe$^{-/-}$ and trJAM-A$^{+/+}$apoe$^{-/-}$ mice were fed for 2 weeks, 6 weeks or 12 weeks to represent different stages of plaque formation. After HFD, isolated complete aorta from each mouse was stained by oil-red-O to visualize plaque area. According to the representative pictures of aortas, the plaque size enlarged progressively with time of HFD (Figure 15A, B, C). The quantification showed that plaque formation in aortic arch was significantly increased after 2 weeks HFD in trJAM-A$^{-/-}$apoe$^{-/-}$ mice (Figure 15D). Similarly, aortic plaque formation in trJAM-A$^{-/-}$apoe$^{-/-}$ mice was significantly enhanced, compared to trJAM-A$^{+/+}$apoe$^{-/-}$ mice fed HFD for 6 weeks and 12 weeks (Figure 15E, F). These results suggest the atheroprotective role of platelet JAM-A in aortic atherosclerosis.

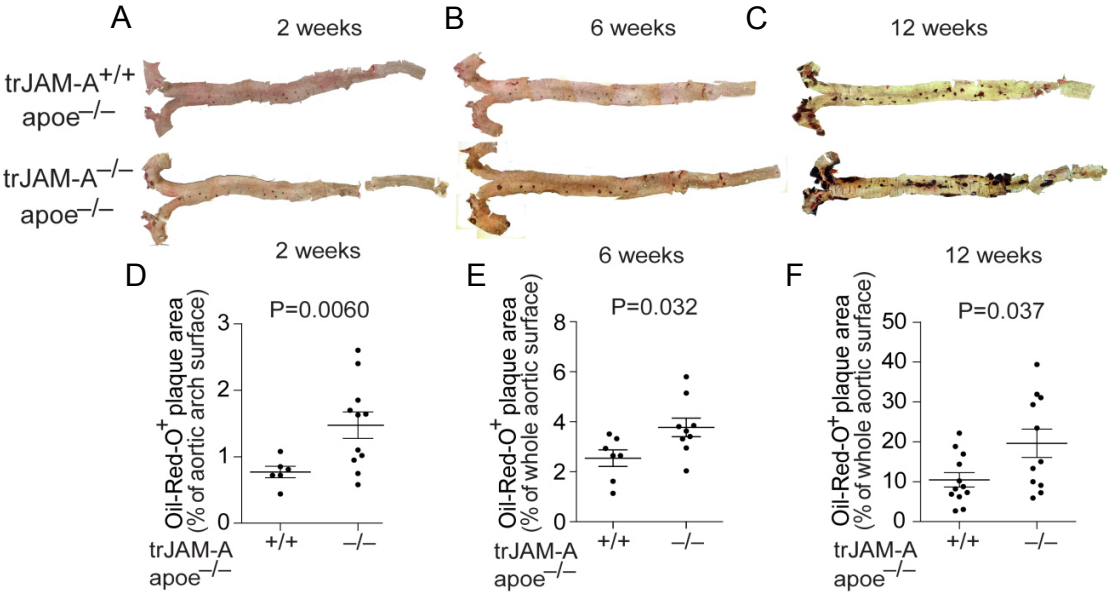


Figure 15: trJAM-A deficiency augmented aortic atherosclerosis. trJAM-A$^{+/+}$apoe$^{-/-}$ and trJAM-A$^{-/-}$apoe$^{-/-}$ mice were fed HFD for 2 weeks, 6 weeks or 12 weeks. Oil-red-o staining visualized plaques in complete aortas (A, B, C). Percentages of plaque areas in aortic arch after 2 weeks HFD (D) and in whole aorta after 6 weeks´ (E) and 12 weeks HFD (F) were quantified. Data represents mean±SEM (N=7-12) and all P values were calculated by Student´s t-test.

4.6.2 Platelet–JAM-A deficiency enhances early-stage atherosclerosis in aortic root

To further verify the effects of JAM-A in atherosclerosis, plaques in aortic roots from trJAM-A$^{-/-}$ apoe$^{-/-}$ and trJAM-A$^{+/+}$apoe$^{-/-}$ mice fed HFD for 2 weeks, 6 weeks or 12 weeks to imitate different stages of atherosclerosis were visualized and quantified. According to the representative pictures and the average size of plaques, hyperlipidemic mice had grown plaques from 2 weeks to 12 weeks in 2 groups (Figure 16). Importantly, trJAM-A deficiency promoted this progression, especially at early stage of atherosclerosis, as the quantification showed significant increase of plaque size in trJAM-A$^{-/-}$ apoe$^{-/-}$ mice compared to trJAM-A$^{+/+}$apoe$^{-/-}$ mice fed HFD for 2 weeks (Figure 16A). However, there was no marked difference after 6 weeks and 12 weeks HDF (Figure 16B, C). These results suggest that platelet hyperreactivity due to JAM-A deletion enhance atherosclerosis, especially at early stage.

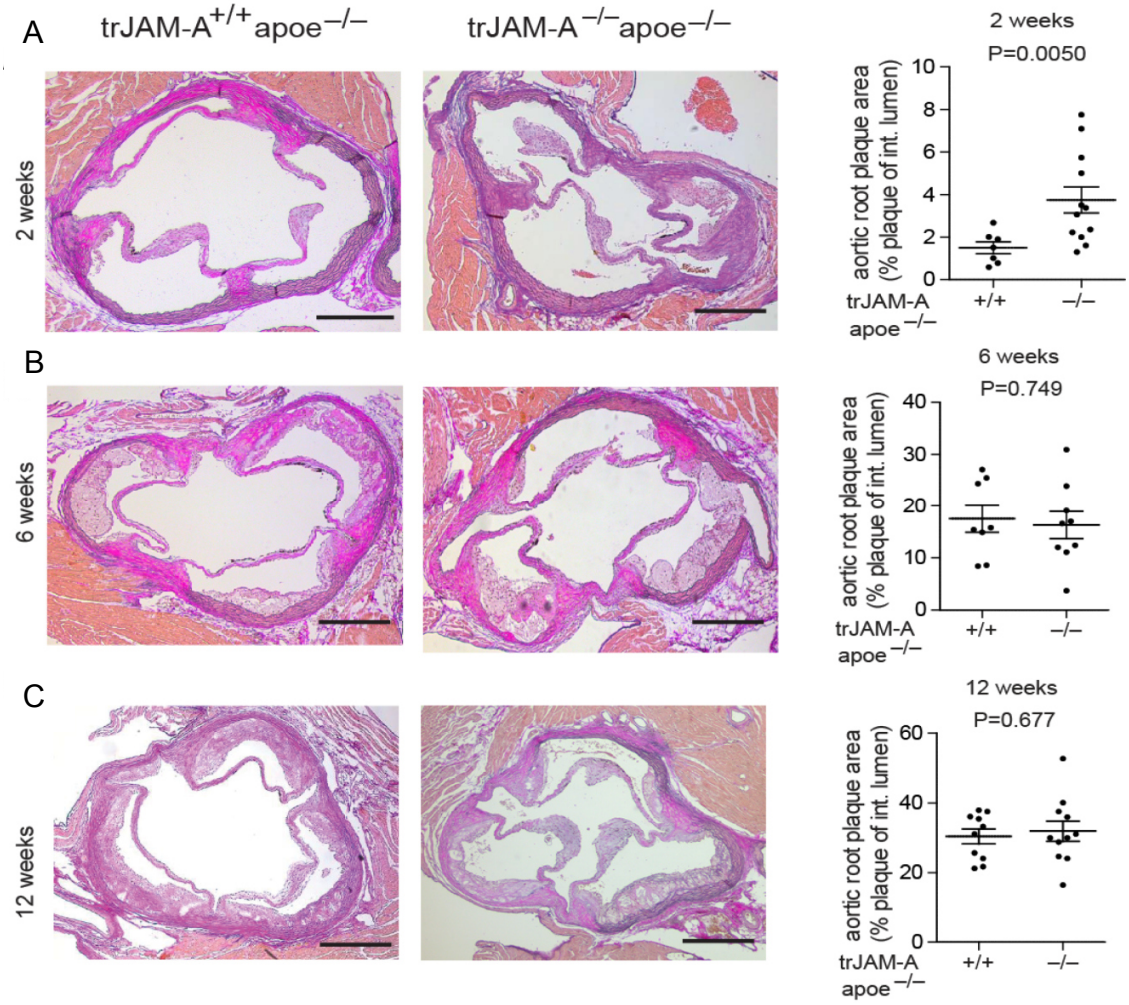


Figure 16: JAM-A–deficient platelets accelerated early-stage plaque formation in aortic root. Representative pictures of EVG staining in aortic root from trJAM-A$^{-/-}$ and trJAM-A$^{+/+}$ mice fed 2 weeks (A), 6 weeks (B) or 12 weeks (C). Scale bar=500µm. Plaque quantification was expressed as size percentage of plaque area in aortic luminal area (mean±SEM). N=7-12 mice per group and all P values were calculated by Student´s t-test.

4.6.3 trJAM-A absence increases leukocyte infiltration into early-stage plaques

The plaque cell composition was analyzed using immunofluorescence staining in aortic roots. The number of MAC-2$^+$ macrophages in early plaques (2 weeks HFD) displayed a marked increase in trJAM-A$^{-/-}$apoe$^{-/-}$ mice, compared to the control group (Figure 17A). Similarly, CD3$^+$ cells staining revealed significantly higher infiltration of T cells in plaques of trJAM-A$^{-/-}$apoe$^{-/-}$ mice, compared with control littermates (Figure 17B). The data suggests that trJAM-A deficiency promotes inflammatory progress at early-stage of atherosclerosis. SMCs (α-SMA$^+$ cells) in plaque significantly increased in aortic roots of trJAM-A$^{-/-}$apoe$^{-/-}$ mice fed 2 weeks HFD (Figure 17C). These increased cell contents in plaques contributed to the enhanced atherosclerosis at early stage.

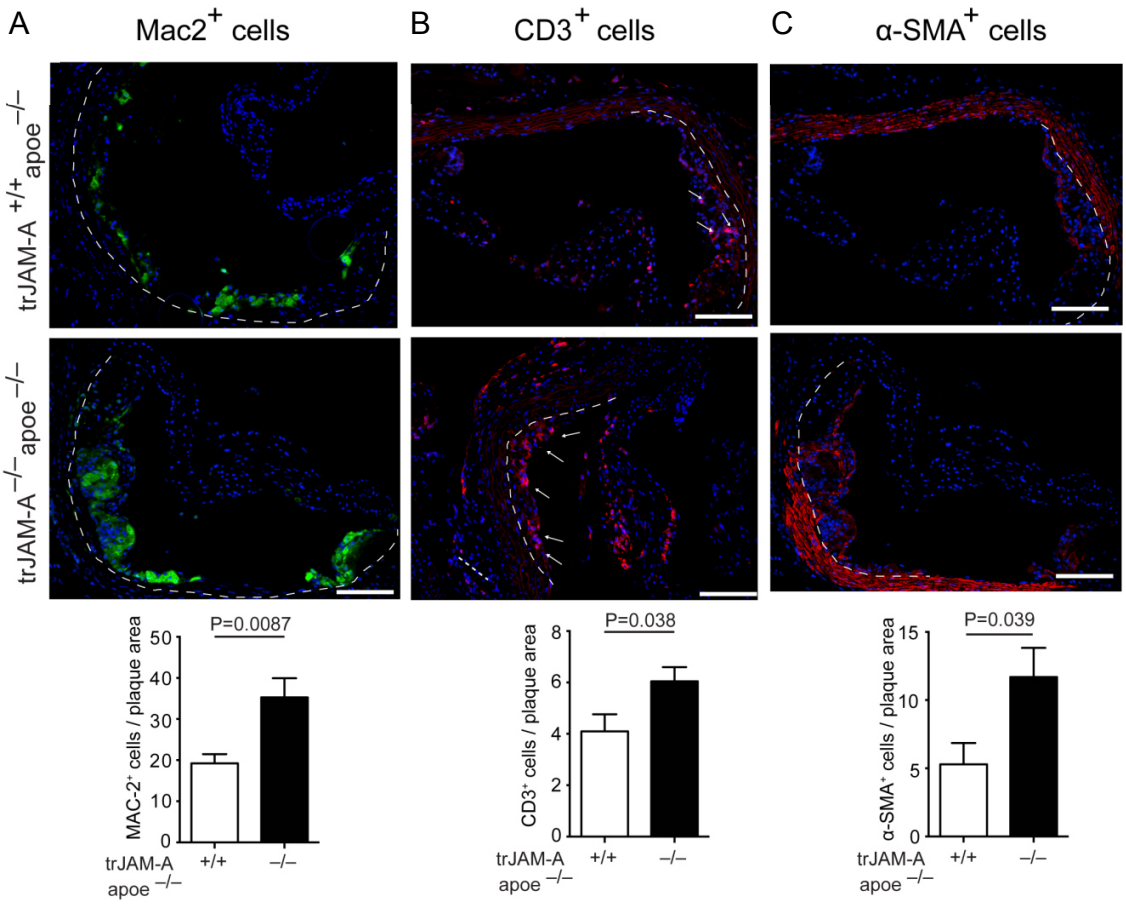


Figure 17: Plaque cell compositions at early stage of atherosclerosis. Immunofluorescence staining of MAC-2 (A, green), CD3 (B, pink) and α-SMA (C, red)

in aortic roots from trJAM-A$^{+/+}$apoe$^{-/-}$ and trJAM-A$^{-/-}$apoe$^{-/-}$ mice fed 2 weeks HFD. Dashed lines represent plaque boundary. Asterisks show aortic lumen and arrows direct CD3 positive T cells. Nuclei (blue) were stained by DAPI. Scale bar=100µm. Quantifications display the positive cells number in plaque area (n=7-12). $P<0.05$ indicates significant difference and all P were obtained by Student´s t-test with (A, C) or without (B) Welch correction. The means ± SEM are shown.

4.6.4 Platelet–JAM-A deficiency does not alter cell composition in advanced plaques

To study whether platelet JAM-A affects plaque phenotype until later stages, the cell composition in plaques was quantified in aortic roots in trJAM-A$^{+/+}$apoe$^{-/-}$ and trJAM-A$^{-/-}$ apoe$^{-/-}$ mice fed 6 weeks or 12 weeks. Platelet JAM-A deletion did not influence Mac2^{+} macrophage and CD3^{+} T cells content in plaques. It indicated that relevance of platelet JAM-A diminished at later stages of atherosclerosis, compared to early inflammatory progress. Additionally, SMCs in plaques also showed no difference in trJAM-A$^{-/-}$ apoe$^{-/-}$ mice versus trJAM-A$^{+/+}$ apoe$^{-/-}$ mice (Figure 18).

Taken together with the observation at early stage (2 weeks), platelet JAM-A inhibited atherosclerosis, especially at initial stages, by suppressing inflammatory cell infiltration into plaques and SMC accumulation in plaques. However, this contribution declined during plaque progression.

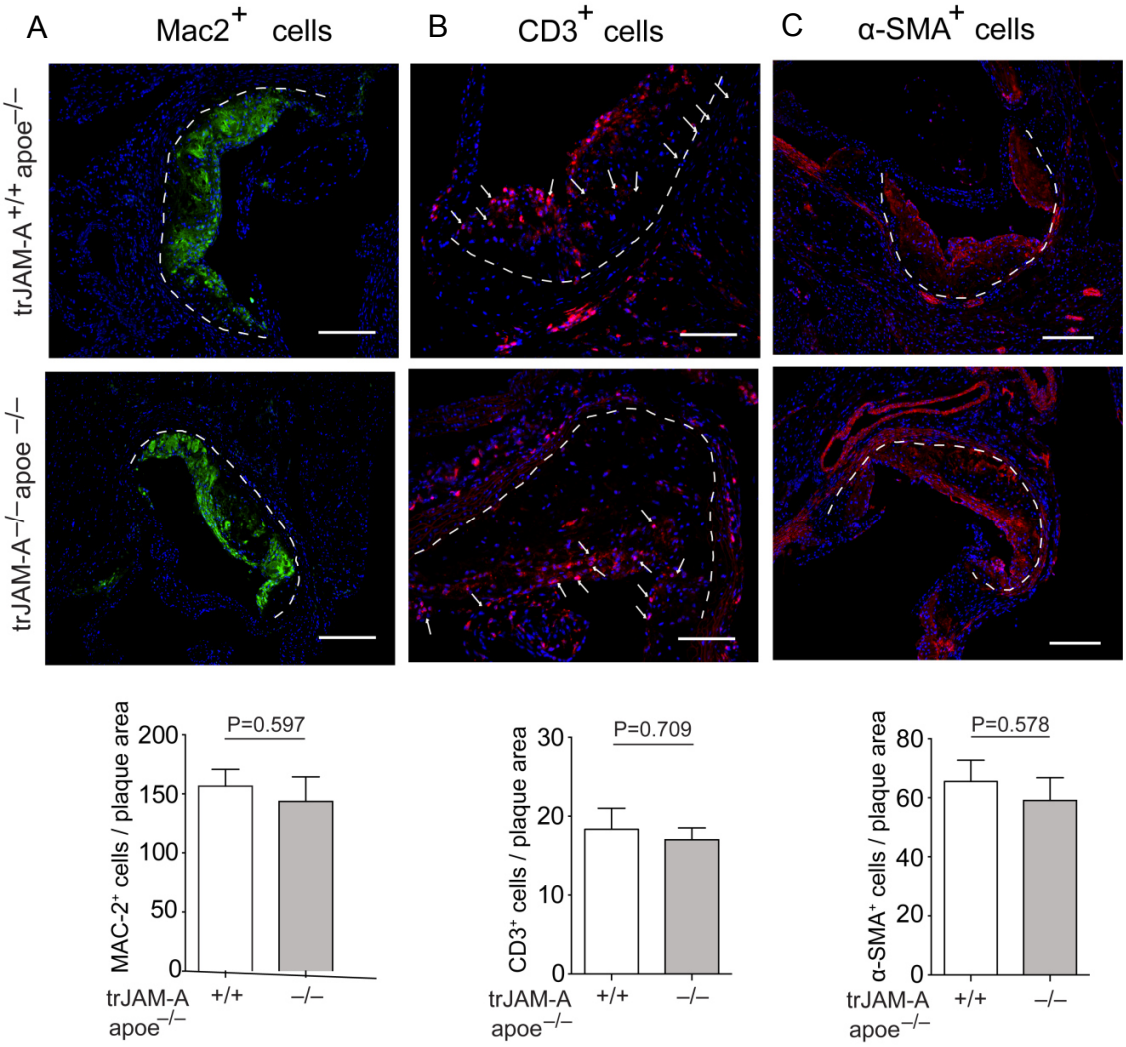


Figure 18: Plaque cell composition in advanced plaque. Immunofluorescence staining of MAC-2 (A, green), CD3 (B, pink) and α-SMA (C, red) in aortic roots from trJAM-A$^{+/+}$apoe$^{-/-}$ and trJAM-A$^{-/-}$apoe$^{-/-}$ mice after 12 weeks. Dashed lines represent plaque boundary. Asterisks show aortic lumen and arrows direct CD3 positive cells. Nuclei (blue) were stained by DAPI. Scale bar=200µm (A, C) and 100µm (B). Quantifications display the positive cells number in plaque area (n=7-12). P<0.05 indicates significant difference and all P were obtained by Student´s t-test. The means ± SEM are shown.

4.7 Platelet–JAM-A deficiency facilitates neointimal formation

It has been found that JAM-A deficiency reduced neointimal lesion formation and monocyte infiltration in atherosclerosis-prone mouse model with somatic JAM-A deletion[39]. But there is no evidence about cell-specific role of platelet JAM-A in vascular remodeling after acute injury. As platelets are the first cells arriving at inflammatory sites[9], it is important to clarify the importance of platelet hyperreactivity in neointimal formation.

4.7.1 JAM-A deficiency encourages platelet adhesion on injured arterial wall

To investigate JAM-A$^{-/-}$apoe$^{-/-}$ platelets adhesion on endothelium-denuded vessel wall, TPLSM was applied to detect platelets adhesion 1 hour after wire-induced injury in carotid arteries ex vivo from trJAM-A$^{+/+}$apoe$^{-/-}$ and trJAM-A$^{-/-}$apoe$^{-/-}$ mice fed 1 week HFD. The adherent platelets on artery wall were constructed into 3-D images and measured as volume in each field. The intact endothelium in carotid artery was shown as CD31 staining (Figure 19A, left), compared to endothelium-denuded artery wall covered by platelets labeled with anti-CD41-PE antibody (Figure 19A, middle and right). The complete endothelium-denuded area (no CD31 staining) for platelets measurement in 3-D image was depicted as schematic diagram from surface and cross-sectional views of artery wall (Figure 19B). JAM-A$^{-/-}$apoe$^{-/-}$ platelets adhesion to the vessel wall increased according to 3-D images, compared to JAM-A$^{+/+}$apoe$^{-/-}$ platelets. Statistic data showed that JAM-A$^{-/-}$apoe$^{-/-}$ platelets adhesion to the endothelium-denuded vessel wall was significantly increased versus JAM-A$^{+/+}$apoe$^{-/-}$ platelets.

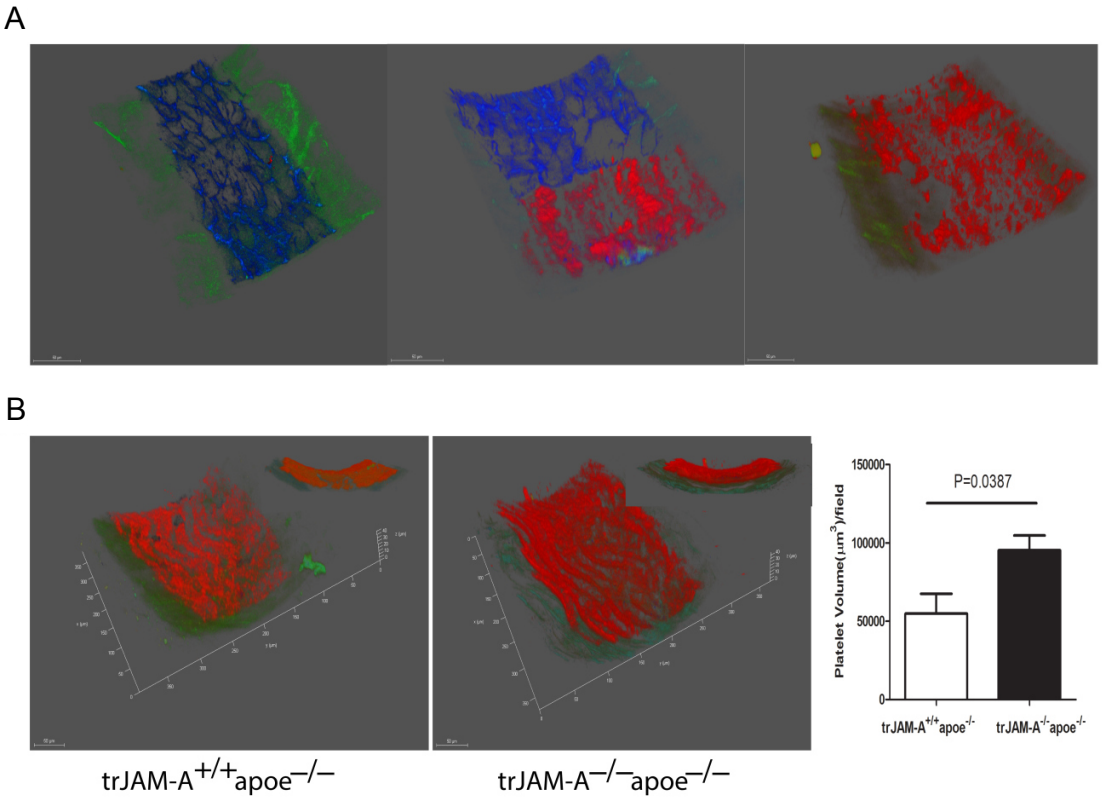


Figure 19: JAM-A deficiency improved platelets adhesion to acute injured vessel wall. 3-D images from HPLSM show normal and endothelium-denuded carotid arteries (A) 1hour after injury in trJAM-A$^{+/+}$apoe$^{-/-}$ and trJAM-A$^{-/-}$apoe$^{-/-}$ mice fed 1 week HFD. Endothelium labeled by anti-CD31-eFluor450 antibody (Blue) and platelets stained by anti-CD41 antibody (Red). Green color is from autofluorescence in the vessel wall. Schematic diagrams from the surface and cross-sectional views of artery represent measurement of adherent platelets (B). Scale bar: 50µm. Quantification of platelet volume (µm^3) in each certain field was showed (>3 fields/mouse). Data: the average volume in each field per mouse expressed as mean±SEM. N=4 mice per group. P<0.05 indicates significant difference and P values were obtained by Student´s t-test.

4.7.2 Promoted neointimal formation at early stage by platelet–JAM-A deficiency

Vascular injury causes endothelial denudation and exposure of extracellular matrix, which is followed by platelet adhesion, thrombus formation, leukocytes infiltration[16], and SMCs proliferation, migration as well as secretion of extracellular matrix[17]. JAM-A deficiency augmented platelets adhesion to the vessel wall after 1 hour injury, which might contribute to neointimal formation at early stage. The neointimal formation after wire-induced injury 2 weeks in the left carotid artery from trJAM-A$^{+/+}$apoe$^{-/-}$ and trJAM-A$^{-/-}$apoe$^{-/-}$ mice was quantified using EVG staining (Figure 20). The data shows significantly increase of neointimal formation in trJAM-A$^{-/-}$apoe$^{-/-}$ mice, compared to the control group, indicating that trJAM-A might suppress neointimal formation at early-stage after vascular injury.

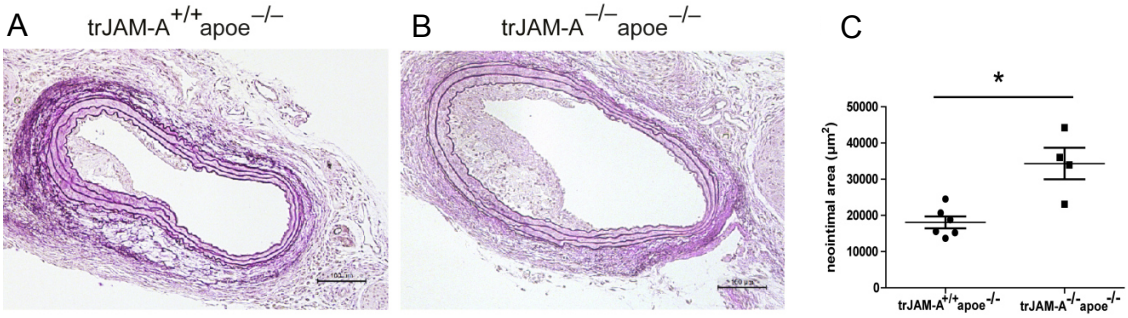


Figure 20: Platelet–JAM-A deficiency increases neointimal formation at early stage. Carotid artery sections from trJAM-A$^{+/+}$apoe$^{-/-}$ and trJAM-A$^{-/-}$apoe$^{-/-}$ mice after wire-injury 2 weeks were visualized by EVG staining to delineate neointimal lesion (A, B). Before 1 week and after 2 weeks of wire-injury, mice were put on HFD. Scale bars: 100 µm. Neointima was measured and average size was expressed as (mean± SEM) µm^2 per mouse (C). N=4-6. *P<0.05. P values were obtained using two-tailed Student's t-test.

4.7.3 Platelet–JAM-A does not affect neointimal formation at advanced stage

Acute endothelial injury will cause the vessel wall remodeling, characterized by neointimal formation. We have found that platelets adhesion increased due to JAM-A deficiency in this acute response. To address whether platelets with JAM-A deficiency promotes neointimal formation continuously, the neointimal size was quantified after wire-induced injury 4 weeks in the left carotid artery from trJAM-A$^{+/+}$apoe$^{-/-}$ and trJAM-A$^{-/-}$apoe$^{-/-}$ mice using EVG staining. The results showed that there was no significant difference between two groups (Figure 21), which indicates that the influence of platelet–JAM-A deficiency was limited at advanced stage of vascular remodeling.

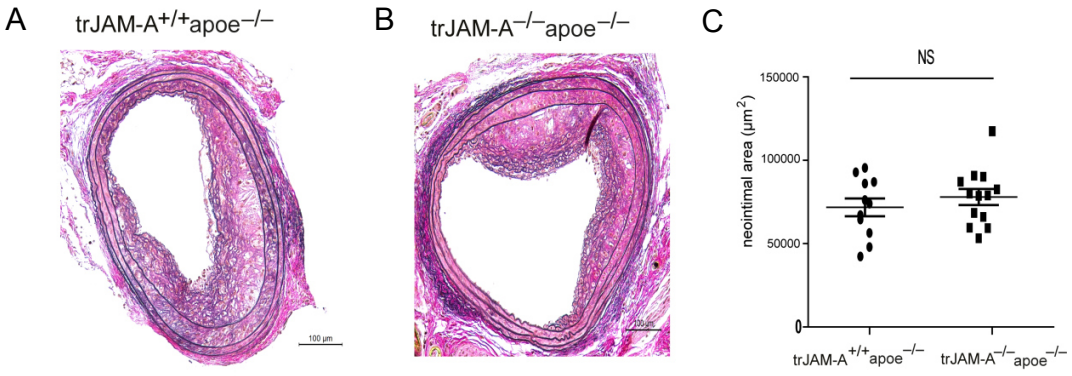


Figure 21: Platelet–JAM-A deficiency did not alter neointimal formation at advanced stage. Carotid arterial sections from trJAM-A$^{+/+}$apoe$^{-/-}$ and trJAM-A$^{-/-}$apoe$^{-/-}$ mice after wire-injury 4 weeks were visualized by EVG staining to delineate neointimal lesion (A, B). Before 1 week and after 4 weeks of wire-injury, mice were put on HFD. Scale bars: 100 µm. Neointima was measured and average size was expressed as (mean± SEM) µm^2 per mouse (C). N=11-13. NS: no statistically significant difference (P>0.05). P values were obtained using two-tailed Student's t-test.

4.7.4 Platelet–JAM-A deficiency mildly affects neointimal cell composition

We investigated the cell composition in advanced neointimal lesion after platelet–JAM-A deficiency. Macrophages and SMCs were visualized by the immunostaining of MAC-2 and α-smooth muscle actin (α-SMA). The quantification of cell number in plaques showed no significant difference (Figure 22A, B). However, the absolute $CD3^+$ cells in neimtina significantly increased after platelet–JAM-A deficiency (Figure 22C). To address the effect of platelet–JAM-A on re-endothelialization after intimal injury 4 weeks, vWF as endothelial cell marker was stained in carotid arteries. The length of vWF positive lines in luminal circumferences was measured. The data showed no significant difference (Figure 22D). Taken together, trJAM-A deficiency mildly enhanced inflammatory cells infiltration into the neointima, especially T cells. The cell composition of neointima at early stage remains to be investigated.

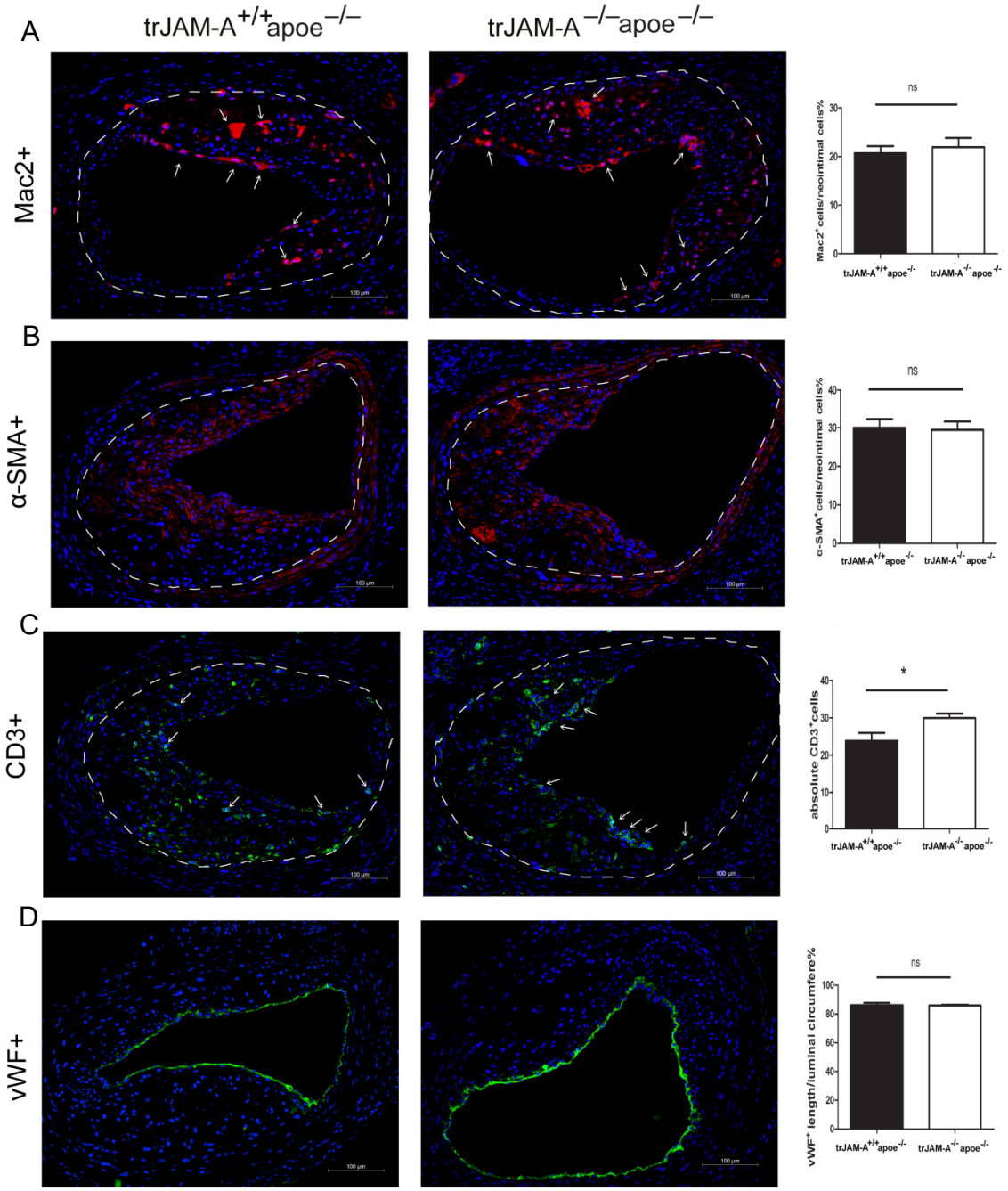


Figure 22: Platelet–JAM-A deficiency mildly alters neointimal cell composition in advanced lesion. Representative pictures and quantifications of MAC-2 (A), α-SMA (B), CD3 (C) and vWF (D) stained in left carotid artery sections after wire-induced injury 4 weeks from trJAM-A$^{+/+}$apoe$^{-/-}$ and trJAM-A$^{-/-}$apoe$^{-/-}$ mice. Neointimal areas were demarcated with dashed lines, MAC2- and CD3- positive cells were marked with arrows.

Nuclei were stained with DAPI (blue). Scale bar=100μm. The quantification of cell composition was expressed as $MAC2^+$ or α-SMA^+ cells in total neointimal cells. Absolute $CD3^+$ cells in neointima were compared. The length percentages of vWF^+ lines in luminal circumferences were calculated. Data: mean±SEM. N=5-8 mice per group.*$P<0.05$ indicates significant difference and P values were obtained by Student´s t-test.

5 Discussion

Atherosclerosis is characterized a chronic inflammatory disease leading to fatal complications such as myocardial infarction, stroke and peripheral artery disease. During disease progression, atherosclerotic plaque is formed by endothelial cell activation, lipid accumulation, inflammatory cell infiltration, macrophage and foam cell formation, SMC and extracellular matrix accumulation in the intima[2, 4]. It is well known that platelet activation plays a substantial role in vascular inflammation and antiplatelet therapy is effective to prevent and treat cardiovascular events[192, 193]. Remarkably, some studies have indicated that platelets can function as initiators and propagators of atherosclerosis[60, 77]. However, there is little evidence to directly illustrate the role of platelet hyperreactivity in vascular inflammation and atherosclerotic formation. So we implemented mouse models with platelet hyperreactivity due to JAM-A deficiency into the context of atherosclerosis, demonstrating platelet-mediated increase of vascular disease. We found that platelet-specific JAM-A (trJAM-A) deficiency caused platelet hyperaggregation under hyperlipidemic conditions. To explore the function of $JAM\text{-}A^{-/-}$ platelets in atherosclerosis, we found increased plasma levels of chemokines and enhanced interactions of platelet-leukocyte and platelet-endothelium in $trJAM\text{-}A^{-/-}apoe^{-/-}$ mice. In turn, elevated recruitment of $JAM\text{-}A^{-/-}apoe^{-/-}$ platelets with leukocytes to inflamed vessel wall was demonstrated and eventually led to aggregated atherosclerotic plaque formation in $trJAM\text{-}A^{-/-}apoe^{-/-}$ mice, particularly in early stages, compared to $trJAM\text{-}A^{+/+}apoe^{-/-}$ mice. Notably, these pro-inflammatory effects of $JAM\text{-}A^{-/-}apoe^{-/-}$ platelets could be abolished by inhibition of integrin $\alpha IIb\beta 3$ signaling *in vitro*.

Despite atherosclerosis, platelets also play pivotal roles in neointimal formation. Activated platelets release cytokines to activate endothelial cells and recruit leukocytes into the injured vessel wall[37], indicating platelet regulated vascular repair progress. Recently, we found that JAM-A has cell-specific role in atherosclerosis[40] and a previous study showed that the globally genetic deletion of JAM-A in apoe-deficient mice decreased neointimal hyperplasia[39], suggesting the role of platelet-specific JAM-A on neointimal formation potentially important. According to our data, platelet–JAM-A inhibited neointimal formation at early stage (2 weeks), but not at advanced stage (4 weeks) after vascular acute injury. The mechanism remains to be clarified.

Taken together, the hyperreactivity of platelets due to JAM-A deficiency promotes vascular remodeling, especially at early-stage.

5.1 Platelet-specific JAM-A knockout

To determinate the efficiency of JAM-A gene knockout in platelet-specific (tr)JAM-A$^{-/-}$ apoe$^{-/-}$ mice[40, 184], we checked the JAM-A expression on platelets, leukocytes as well as in vessel wall (Figure 5,6 and additional data referred to our paper) and found that platelet JAM-A was specifically deleted in trJAM-A$^{-/-}$apoe$^{-/-}$ mice without affecting JAM-A expression on leukocytes and SMCs. After JAM-A deficiency in platelets, JAM-C and ESAM expression was not altered (Figure 7), suggesting remaining functions of JAM-A$^{-/-}$apoe$^{-/-}$ platelets unaffected. Additionally, mean platelet volume (MPV) showed that platelet production was not altered, as same as leukocyte numbers in blood of trJAM-A$^{-/-}$apoe$^{-/-}$ mice (Table 1). Importantly, there was no significant difference in cholesterol and triglyceride levels between trJAM-A$^{-/-}$apoe$^{-/-}$ mice and the control group, indicating that platelet JAM-A might not affect lipid metabolism. These data revealed that platelet-specific JAM-A deficiency did not influence the homeostasis in our mice model, which rendered the reliability to investigate the role of platelet–JAM-A in vascular remodeling.

5.2 JAM-A–deficient platelets gain hyperreactivity

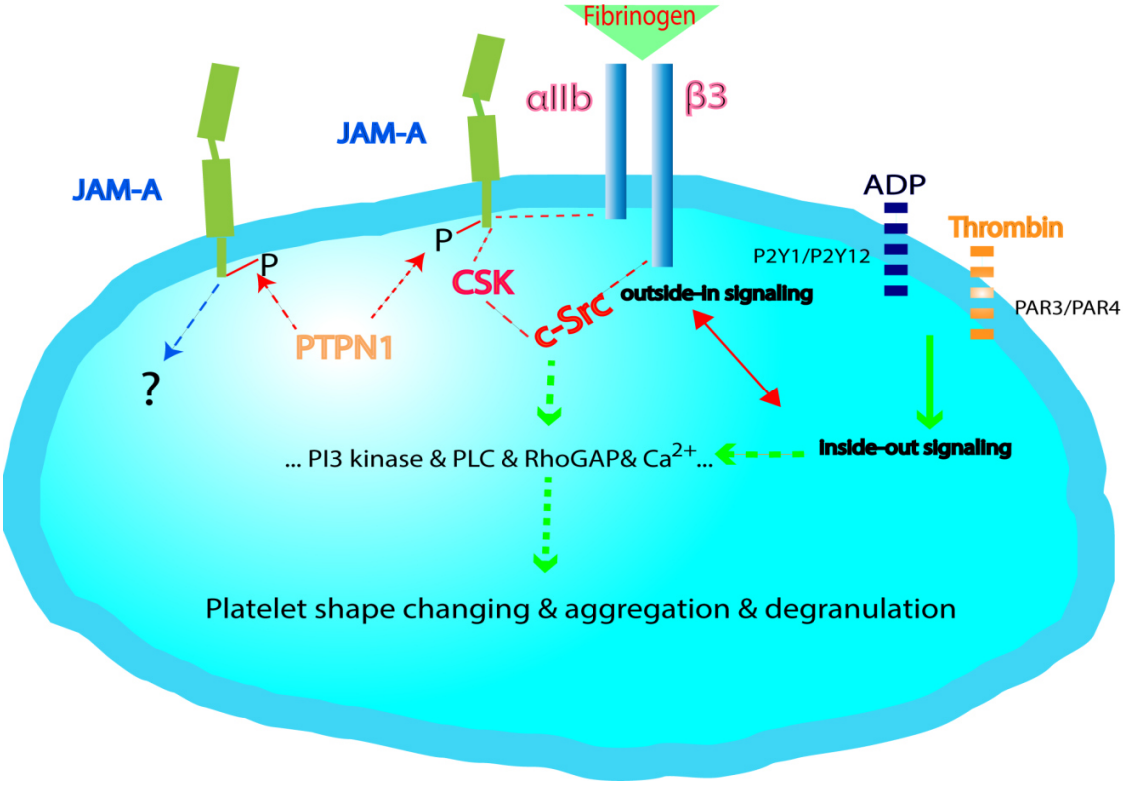


Figure 23: JAM-A inhibiting platelet activation via integrin αIIbβ3 signaling. In resting platelets, one complex is formed as CSK–JAM-A–αIIbβ3–c-Src on the cell membrane. JAM-A is phosphorylated and recruits CSK to this complex to inhibit c-Src activation in cytoplasmic side. After platelet activated by stimuli, such as ADP via P2Y1/P2Y12 in mice and thrombin via PAR3/PAR4, the inside-out signaling is triggered and induces integrin αIIbβ3 activation for fibrinogen binding, which initiates outside-in signaling. Consequently, JAM-A and CSK dissociate from the complex and c-Src is activated. After dissociation, JAM-A is dephosphorylated by PTPN1and transduces unknown signals into platelet. The activated inside-out and outside-in signaling eventually result in platelet shape change, aggregation as well as degranulation via PI3 kinase-, PLC- and RhoGAP-mediated pathways with increased cytoplasmic levels of $Ca2^{+}$. JAM-A: Junctional adhesion molecular-A. c-Src: Tyrosine-protein kinase. CSK: c-src tyrosine kinase. PTPN1: Tyrosine-protein phosphatase non-receptor type 1. (This pathway bases on the findings of Naik et.al[181])

Discussion

Platelets are important player in chronic pathological processes including the atherosclerotic remodeling[9]. The interaction of platelet receptors with adhesion molecules, such as vWF recptor–GPIb/V/IX and collagen receptor–GPVI, facilitates platelet adhesion and activation at sites of inflamed vascular wall[194], which in turn induces inside-out activation of integrins to strengthen the adhesion, including integrin αIIbβ3 (GPIIb/IIIa) or α2β1 (GPIa/IIa)[194, 195]. Once the initial signals have been triggered, platelets will release a variety of mediators to amply platelets activation, such as ADP, and thromboxane A_2. Interestingly, on the surface of activated platelets, the formation of mature thrombin is triggered and further promotes platelet activation[196]. These diffusible mediators binding to G-protein-coupled receptors (GPCRs), involving 3 major G proteins Gq, G13 and Gi, amplify the initial signals[197]. ADP stimulates platelet intracellular signaling through G protein-coupled receptors, P2Y1 and P2Y12, whereas thrombin via protease-activated G-protein-coupled receptors PAR1/PAR4 (human) or PAR3/PAR4 (mouse), resulting in platelet shape change, degranulation and multiple platelet/platelet interactions (platelet aggregation)[197, 198]. Platelet aggregation is mediated by integrin αIIbβ3, which binds various extracellular macromolecular ligands, such as fibrinogen and vWF. Signals transduced by αIIbβ3 regulate platelet activity, such as shape change, aggregation and granule secretion[199-201].

JAM-A deficiency augments platelet aggregation. We used ADP and thrombin at different concentrations to investigate platelet activation. JAM-$A^{+/+}$apoe$^{-/-}$ platelet activation at low concentrations of thrombin showed no difference (Figure 8C), which might be due to the cofactor-assisted PAR activation on platelets, consistent with previous observations[202]. Notably, low concentrations of thrombin dramatically stimulated JAM-$A^{-/-}$apoe$^{-/-}$ platelet aggregation compared with JAM-$A^{+/+}$apoe$^{-/-}$ platelets. This result was similar with ADP stimulated platelets (Figure 8). Thus, JAM-A deficiency caused platelets to be easily activated by agonists. Naik and coworkers found that genetic ablation of JAM-A in mice enhanced thrombotic function of platelets in vivo with increased platelet aggregation[182]. Taken together, JAM-A inhibits platelet function, analogous to other adhesion molecules such as endothelial cell-selective adhesion molecule (ESAM), which down-regulated platelet aggregation and thrombus

formation[203]. In addition, CD31 on platelets suppressed platelet aggregation as its crosslinking reduced agonist-induced platelet aggregation[204] and genetic deletion of CD31 in platelets, not in endothelial cells, increased thrombus formation *in vivo*[205]. After CD31 activation by vWF binding to GPIb/IX/V complex, as a negative feedback loop, CD31 in turn suppresses GPIb-initiated platelet signaling responses, thus inhibiting the extent of platelet activation and aggregation[206].

It has been found that JAM-A regulates platelet activity through integrin αIIbβ3 outside-in signaling[182]. In our study, tirofiban as a specific antagonist to integrin αIIbβ3 reversed the hyperaggregation of JAM-A$^{-/-}$apoe$^{-/-}$ platelets to the level of JAM-A$^{+/+}$apoe$^{-/-}$ platelets (Figure 9). Thus, our data also confirm that JAM-A regulates platelet reactivity by inhibiting integrin αIIbβ3. Previous researches showed that some specific protein tyrosine kinases and phosphatases might be involved in the downstream of signal pathway in platelet activity. Especially, Src family kinases have been found pivotal in outside-in signaling in many cell types[207-210]. Thus, c-Src tyrosine kinases activity was assessed in our experiments, as it is well known that c-Src binds constitutively and selectively to β3 in platelets[178, 211]. Notably, PP2, as an inhibitor of c-Src, reversed the hyperreactivity of JAM-A$^{-/-}$apoe$^{-/-}$ platelets effectively (Figure 10A, B). Furthermore, phosphorylation degree of Tyr-418 in the c-Src loop reflects the extent of c-Src activation in integrin outside-in signaling[211]. We used soluble thrombin to trigger inside-out signaling (without outside-in signaling stimulation) in isolated platelets and found that the extent of c-Src Y418 phosphorylation was not affected by JAM-A deficiency (Figure 10C, D). This data support that c-Src and JAM-A might be not involved in integrin inside-out signaling. Conversely, c-Src Y418 phosphorylation was elevated in JAM-A$^{-/-}$ apoe$^{-/-}$ platelets after Fg stimulation, which triggers outside-in signaling (Figure 10E, F). Taken together, c-Src as the signal molecule transduces outside-in signaling via integrin αIIbβ3, which is inhibited by JAM-A in platelets.

JAM-A suppresses c-Src activity on platelets[182]. However, in resting JAM-A$^{+/+}$apoe$^{-/-}$ and JAM-A$^{-/-}$apoe$^{-/-}$ platelets, there was no significant difference of c-Src Y418 phosphorylation (Figure 10D, F), indicating that c-Src activity was not stimulated solely

due to JAM-A deficiency in resting platelets. In literature, c-Src can become partially activated in cell resting state, but not fully activated, after binding to the β3 integrin tail through Src homology 3 (SH3) domain[211]. Additionally, intramolecular interactions of SH3 binding to Src homology 2 (SH2) as well as other kinase domains regulate Src conformation and auto-inhibit Src activity[212, 213]. It has been shown that SH3 binding to integrin β3 tail in activated platelets disrupts fully intramolecular auto-inhibition of Src SH3 and increases Src activity[211, 214, 215]. Furthermore, full activation of c-Src requires ligand binding to β3 integrin, together with JAM-A dissociation from integrin αIIbβ3[30]. To determinate how JAM-A suppresses c-Src activation initially described by Naik *et al.*, they found the endogenous c-Src inhibitor CSK (C-terminal Src kinase) in the complex of integrin αIIbβ3–c-Src. Importantly, tyrosine-phosphorylated JAM-A recruits CSK binding to the complex, which in turn phosphorylates c-Src Y529 residue to constrain c-Src activation[178, 181]. After ligand binding to integrin αIIbβ3, JAM-A and CSK dissociates from αIIbβ3, resulting in c-Src auto-phosphorylation for the transduction of outside-in signaling[211].

JAM-A was found tyrosine-phosphorylated in resting platelets (data shown in our paper), consistent with other studies[179, 181], which gave the possibility of JAM-A interaction with its intracellular binding partner. In the present study, JAM-A was de-phosphorylated after platelet activated by Fg, which was inhibited by broad range protein tyrosine phosphatases (PTPs), indicating that PTPs are responsible for JAM-A de-phosphorylation. Furthermore, we found that JAM-A de-phosphorylation was blocked by specifically inhibiting the tyrosine phosphatase PTPN1 (PTP1B), but not PTPN6 and PTPN11 (Figure 11A, B). Both in resting and activated platelets, JAM-A appeared to be associated with PTPN1 (Figure 12A), which can be recruited to integrin complex after integrin αIIbβ3 activation[187]. Additionally, Naik *et al.* have found that JAM-A is close proximity to the integrin αIIbβ3 complex. Therefore, PTPN1 is the phosphatase responsible for JAM-A de-phosphorylation after platelet activation, which might be a novel aspect of JAM-A regulating integrin outside-in signaling. However, the role and fate of de-phosphorylated JAM-A after platelet activation remains to be elucidated.

Besides its interaction with integrin αIIbβ3, JAM-A regulates platelet activation through other mechanisms that are still unclear. In the present study, JAM-A deletion lowers the activation thresholds of integrin αIIbβ3 and elevates platelet activation state, which promotes vascular inflammation and atherosclerosis eventually. Integrin outside-in signaling can reinforce platelet aggregation by intracellular signaling cascade, which is triggered by platelet activation stimuli, such as ADP and thrombin, as a feedback loop of signaling pathways initiated by G protein-coupled receptors and integrins[216]. However, the mechanism of platelet–integrin αIIbβ3 regulating inflammatory response remains to be investigated. It has been shown that αIIbβ3 interaction with other integrins, such as GPVI, regulates platelet activation[217]. Non-specific blocking integrins, including αIIbβ3, showed long-term anti-inflammatory effects by suppressing the rise of circulating inflammatory markers, such as C-reactive protein and IL-6[218]. In the present study, tirofiban, as a specific inhibitor of αIIbβ3, suppressed platelet aggregation and adhesion to ECs. Conversely, it has been found that αIIbβ3 antagonists with active conformation inhibit inflammation by blocking platelet aggregation and reduce release of inflammatory mediators, such as soluble CD40 and CCL5[219, 220]. The different effects of integrin ligands might be dependent on their concentrations. Moreover, genetic αIIbβ3 in mice deficiency inhibited platelet adhesion to injured or atherosclerotic vessel wall and attenuated lesion formation[221]. Additionally, blocking αIIbβ3 using specific antibody or depletion of platelets using antiserum also reduced intimal hyperplasia, highlighting the pivotal role of platelet αIIbβ3 in vascular inflammation and remodeling[222]. A cohort study showed that a single nucleotide polymorphism (SNP) of αIIbβ3 increased risk of atherosclerotic plaque rupture[223]. In contrast to the augmenting the role of platelet αIIbβ3 in atherosclerosis, Glanzman thrombasthenia patients with platelet αIIbβ3 deficiency did not show decreased atherosclerosis, despite intact functions of platelet adhesion and release of granule contents in response to adequate stimuli[224]. Together with the present study, JAM-A deficiency caused platelet hyperreactivity through up-regulated integrin αIIbβ3 signaling, which enhanced vascular inflammation and atherosclerosis. Thus, JAM-A might inhibit platelet activity by suppressing integrin αIIbβ3 outside-in signaling to regulate vascular remodeling (Figure 23).

5.3 JAM-A deficient platelets propagate vascular inflammation

Various substrates and soluble mediators activate platelets under blood shear stress, such as oxLDL. Activated platelets upregulate adhesion molecules, such as GPIb/IX/V, P-selectin, CD40, and release pro-inflammatory stimuli deposited on vascular cell surfaces, such as CXCL4 and CCL5. These inflammatory mediators facilitate leukocyte recruitment and modulate the pathogenesis of vascular diseases. Additionally, platelets can trigger chemokine secretion from different cells of vascular wall, which in turn enhance platelet aggregation and adhesion. Thus, platelets have been considered as potent inflammatory cells that propagate inflammatory responses in vascular remodeling[225-227].

5.3.1 Platelet adhesion to atherogenic vessel wall is enhanced by JAM-A deficiency

The hyperreactivity of JAM-A$^{-/-}$apoe$^{-/-}$ platelets was reflected by increased adhesion to endothelium. Consistent with previous studies, we chose the carotid bifurcation as the pro-atherogenic site to be perfused with whole blood and then quantified platelet adhesion on endothelium by TPLSM *ex vivo*. The data represent that trJAM-A deficiency increased platelet adhesion to the vessel wall, compared to JAM-A$^{+/+}$apoe$^{-/-}$ platelets, which was inhibited by blocking integrin αIIbβ3 (Figure 12). Furthermore, cultured endothelial cells were activated by TNFα and perfused with thrombin-activated platelets, showing that JAM-A deficiency dramatically boosted platelet binding to inflamed endothelium (*Karshovska E, Zhao Z et al, Circ Res. 2015 Feb. 13; 116(4):587-99*). Taken together, JAM-A deficiency augments platelet adhesion to inflammatory and atherogenic vessel wall.

Platelet-endothelial cell interaction is mediated by adhesion molecules and some soluble mediators in plasma, including P-selectin to PSGL-1, GPIb to P-selectin or vWF, and integrin αIIbβ3 to endothelial ICAM-1. For example, integrin αIIbβ3 as an adhesion molecule mediates activated platelet arrest to ICAM-1 or αVβ3 on endothelium, through a bridge formed by Fg, vWF or fibronectin[94]. Integrin αIIbβ links platelets to injured sites in vessels also by interaction with Fg and vWF[88]. Notably, blocking αIIbβ3 (by tirofiban) normalized the hyperadhesion of JAM-A$^{-/-}$ platelets, indicating that αIIbβ3 is

important for platelet adhesion, but it did not significantly affect JAM-A$^{+/+}$apoe$^{-/-}$ platelets adhesion (Figure 12C, D), suggesting that other adhesion molecules are also involved in platelet-endothelial interaction, such as GPIbα[94, 228].

Dysregulation of platelet-endothelial cell interaction was always found at atherosclerotic predilection sites, such as bifurcation of the carotid artery, where plaques develop earlier than at other sites of vessel wall[9]. JAM-A deficiency enhanced this process, as JAM-A$^{-/-}$ platelet adhesion to atherogenic vessel wall and inflamed endothelium was increased, which would further promote the pathophysiology of vascular remodeling[228, 229]. Adherent platelets then activate endothelial cells to express various inflammatory mediators, such as IL-1β, CCL2, macrophage inflammatory protein-1α and ICAM-1[230]. In addition, activated platelets upregulate CD40 ligands, which initiate endothelial inflammation through cognate receptor CD40 and stimulate endothelial cells to express inflammatory mediators[100, 185, 231, 232]. The inflamed endothelium generates crucial signals for leukocyte recruitment and extravasation at sites of vascular injury or initiating an inflammatory response of previously intact endothelium[100, 230]. Interestingly, CD40L on activated platelets can bind to αIIbβ3 triggering outside-in signaling in inflammatory process[231, 233, 234], which might be enhanced by JAM-A absence. Conversely, endothelial cells also can stimulate platelets during inflammatory progress. For example, ADAM15 expressed on endothelium can serve as an adhesion receptor for platelets via integrin αIIbβ3 binding, which in turn leads to platelet activation as well as secretion and promotes thrombus formation in cardiovascular disease[235]. Whether endothelium in trJAM-A$^{-/-}$apoe$^{-/-}$ mice augmented platelets activation via integrin αIIbβ3 in our model remains to be demonstrated. Briefly, JAM-A$^{-/-}$apoe$^{-/-}$ platelet adhesion to endothelial cells significantly increased, compared to JAM-A$^{+/+}$apoe$^{-/-}$ platelets (Figure 12), which might be responsible for increased atherosclerotic plaques in trJAM-A$^{-/-}$apoe$^{-/-}$ mice. Direct interactions of platelets with endothelial cells results in up-regulation of inflammatory mediators and exacerbates vascular inflammation.

5.3.2 Plasma chemokine concentrations increase due to platelet–JAM-A deficiency

Upon activation, platelets release several pro-inflammatory mediators including chemokines, which can modulate endothelial cell activation and leukocytes recruitment to inflammatory sites in the vasculature. In trJAM-A$^{-/-}$apoe$^{-/-}$ mice, we found diminished granular staining of chemokines CXCL4 and CCL5 in platelets, which were paralleled with increased levels in plasma during the time course of HFD administration (Figure 13). These results suggest that increased circulating chemokines might be released from JAM-A$^{-/-}$apoe$^{-/-}$ platelets during the time course of HFD, hinting JAM-A$^{-/-}$apoe$^{-/-}$ platelets at decreased activation threshold of granular release. More chemokines would be deposited onto the vessel wall in trJAM-A$^{-/-}$apoe$^{-/-}$ mice and lead to increased recruitment of leukocytes into plaques. Actually, lots of chemokines have been found in early and advanced atherosclerotic lesions, suggesting that persistent activation of platelets and their secretion contribute to the evolution of cardiovascular diseases[9, 185, 236, 237].

Leukocytes and endothelial cells are the first cells to be exposed to the chemokines released from platelets. Deposition of chemokines from platelets to their cognate receptors on vascular cell surfaces links platelet activation to the recruitment of immune cells and exacerbates vascular diseases[88, 227, 238]. JAM-A deficiency increased plasma levels of CCL5 and CXCL4, which can be delivered to endothelium and leukocytes/monocytes. The effective deposition of chemokines on cell membrane is facilitated by direct interactions of cells or by transfer from platelet microparticles to the vessel wall[60, 239]. As a result, chemokines binding to chemokine receptors (GPCRs) or GAGs on vascular cell surfaces leads to their immobilization and gradients formation for leukocytes recruitment[122]. Thus, we predicted that plasma levels of CCL5 and CXCL4 might enhance leukocytes recruitment into plaques of trJAM-A$^{-/-}$apoe$^{-/-}$ mice.

CXCL4 is stored predominantly within platelets and released upon platelet activation, which can be localized in atherosclerotic lesions and correlated with lesion severity as well as symptomatic atherosclerosis[129]. CXCL4 is known to promote atherosclerosis in CXCL4-deficient mice, which showed significant reduction of atherosclerotic plaques in the aorta[240]. Under conditions of arterial injury or possible endothelial dysfunction with

platelets attachment, CXCL4 is transferred rapidly into the media[241]. Then, CXCL4 stimulates SMCs into an inflammatory phenotype, such as decline of differentiation markers, increase of cytokine production and cell proliferation, which promotes lesion formation after partial ligation of carotid artery[242]. In this study, we suggested that the increased plasma CXCL4 might contribute to the increased accumulation of SMCs at early-stage of plaque formation in trJAM-A$^{-/-}$apeo$^{-/-}$ mice. Although lacking of a chemotactic activity for neutrophils[243] and monocytes[130], CXCL4 initiates a signal transduction cascade leading to a broad spectrum of functions. On endothelium, CXCL4 binds to endothelial cells via cell surface GAGs, promoting leukocyte adhesion by increasing expression of adhesion molecules. In the presence of appropriate costimuli, such as TNF-α, CXCL4 can activate neutrophils, cause exocytosis and promote adhesion to endothelium[125, 128, 244]. On monocytes, CXCL4 has a number of different effects, including anti-apoptosis effect and facilitating macrophage differentiation during inflammatory process[131], increasing monocyte phagocytosis[130] and inducing cytokines secretion[134]. For example, CXCL4 triggers many signaling pathways in monocyte survival and cytokine expression, controlled by Erk and JNK[134].

CCL5 has been found to cause the selective migration of human blood monocytes and T cells via CCR5 and CCR1 receptors[138]. Immobilized and presented on activated endothelium, CCL5 enhances monocyte recruitment[81] and contributes to inflammatory response in vascular remodeling[88]. Conversely, inhibition of CCL5 results in decreased lesion size, both in atherosclerosis and vascular injury[38, 60, 81, 149, 245]. Thus, the present data indicates that increased levels of CCL5 due to platelet–specific JAM-A deficiency propagated vascular remodeling. Plasma CCL5 concentration in trJAM-A$^{-/-}$apoe$^{-/-}$ mice was significantly increased during 2 weeks or 6 weeks HFD feeding (Figure 13D). Notably, at early stage, we found that the abundance of macrophages and T cells in plaques of trJAM-A$^{-/-}$apoe$^{-/-}$ mice significantly increased, compared to JAM-A$^{+/+}$apoe$^{-/-}$ mice, which might be partly caused by the dramatically increased CCL5 in plasma enhancing leukocytes infiltration. However, at a more advanced stage of atherosclerosis, there was no significant difference of plaque size in aortic root and leukocyte contents in plaques between trJAM-A$^{-/-}$apoe$^{-/-}$ mice and trJAM-A$^{+/+}$apoe$^{-/-}$ mice, paralleled with no difference of plasma CCL5. Thus, the role of JAM-A–deficient platelets at advanced

stages of atherosclerosis was limited, which might be reflected by the decreased plasma CCL5 concentration.

Interestingly, CCL5 and CXCL4 concentrations in trJAM-A$^{-/-}$apoe$^{-/-}$ mice showed different trends in the progress of atherosclerosis. Plasma CXCL4 levels in trJAM-A$^{-/-}$ apoe$^{-/-}$ mice increased continuously during HFD, different with the increased plasma levels of CCL5 only at early stages (Figure 13). It indicates that chemokines are released from activated platelets selectively according to different stages of inflammatory disease. In fact, several studies have reported differential, stimulus-specific packaging and release of platelet granules[246, 247], which could potentially enable platelets to specifically release inflammatory granules[248]. For example, Italiano et al. reported differential release of the angiogenic factor vascular endothelial growth factor and endostatin after targeted stimulation of PAR1 and PAR4, respectively[100]. CXCL4 and CCL5 can interact and form a heterodimer. This complex in atherosclerosis has a more potent effect on monocyte arrest to stimulated endothelium than each chemokine alone[133]. Disrupting the CXCL4/CCL5 interactions by using a peptide inhibitor decreases monocyte recruitment and leads to a reduction of atherosclerotic lesions. Taken together, these studies demonstrate a synergic effect on monocyte recruitment by the CXCL4/CCL5 heterodimer complex[249]. We predicate that the increased plasma levels of CXCL4 and CCL5 in trJAM-A$^{-/-}$apoe$^{-/-}$ mice might form more CXCL4/CCL5 complexes, which in turn reinforce leukocytes recruitment.

5.3.3 Leukocyte recruitment is augmented due to JAM-A–deficient platelets

Beside release of increased chemokines driving leukocytes recruitment, JAM-A–deficient platelets also facilitated leukocytes interactions with vessel wall. *In vitro and ex vivo* experiments, we found that JAM-A–deficient platelets showed significantly enhanced adhesion, paralleled with increased co-localization of leukocytes on the pro-atherosclerotic and inflamed endothelium. The augmented firm arrest of monocytes was further demonstrated *in vivo* experiments *(Karshovska E, Zhao Z et al, Circ Res. 2015 Feb. 13; 116(4):587-99)*, which could be explained by previous studies that platelets 'carpet' the endothelium during inflammation to form a 'physical bridge' via P-selectin-dependent mechanisms to promote leukocytes recruitment to endothelium[250-252]. The

mechanisms of platelet adhesion to endothelium for leukocyte recruitment have been investigated. For example, plenty of P-selectin and other adhesion molecules are up-regulated on the platelet membrane after activation, covering inflamed endothelium. Presented selectins promote not only the tethering and rolling of PSGL-1-expressing leukocytes, but also of additional platelets, thus amplifying the recruitment[253-255]. In fact, platelet-derived P-selectin, not endothelial P-selectin, has been identified as predominant ligand for leukocyte recruitment during the inflammatory process[256-258]. Blocking platelet interactions with endothelium and leukocytes by removal of platelet P-selectin effectively delays the onset of atherosclerotic disease[259] and reduces neointimal formation after vascular injury[260]. The following step is firm adhesion and activation of leukocytes via Mac-1 to platelet GPIbα or to integrin αIIbβ3 in a fibrinogen-dependent manner, which in turn triggers release of pro-inflammatory mediators from adherent platelets, including CXCL4 and CCL5[261]. Thus, the up-regulated activity of platelet–integrin αIIbβ3 due to JAM-A deficiency could increase the interaction of platelets with leukocytes, resulting in exacerbated recruitment of leukocytes.

Additionally, JAM-A-deficient platelets showed significantly increased aggregation to leukocytes, as more complexes of platelet-monocyte and platelet-neutrophil in blood, which was abolished by blocking platelet–integrin αIIbβ3 (Figure 14). The increased aggregation of leukocytes with JAM-A–deficient platelets could promote leukocyte rolling and arrest[253-255]. Previous studies have shown that these complexes are relevant with inflammatory diseases and cardiovascular diseases[262-264]. Platelet interaction with leukocytes primarily occurs in a P-selectin/PSGL-1 dependent manner, and is bidirectional. Activated platelets induce activation of transcription factor NF-kB, leukocyte integrins and related gene products in leukocytes[103, 116, 265], which amplify vascular inflammation. We found that platelet-neutrophil complexes in the blood of trJAM-A$^{-/-}$apoe$^{-/-}$ mice were dramatically increased. In previous studies, these complexes may be short-lived, but induce signaling to activate neutrophil integrins, promote the degranulation, enhance phagocytosis[266, 267], and boost neutrophil transmigration across endothelium[268-270]. Additionally, platelets prime neutrophils for efficient adhesion to endothelium via the up-regulation of integrins, such as Mac-1 and enhance responsiveness to chemokines[124, 125], which are required for the recruitment of

neutrophils to inflamed tissue. Activation of the integrin Mac-1 is known to be critical for mediating neutrophil adhesion and migration, involving the protein kinase C zeta (PKCζ)[102]. Beside recruitment promotion, activated platelets have been shown to inhibit neutrophil apoptosis[271], which may further enhance inflammatory roles of neutrophils in vascular disease. Remarkably, it has been clearly found that the interaction of activated platelets with neutrophils in circulation as well as on vessel wall, by the dynamic reorganization of neutrophil domains and receptors, initiates inflammatory responses[272].

Platelet-monocyte aggregates have been shown to be an early predictor for cardiovascular events[105]. The monocyte–platelet complexes form more abundantly, more rapidly and more stable than other platelet-leukocyte complexes. Despite P-selectin–dependent adhesion of platelets to monocytes, it has been inferred that PSGL-1/P-selectin interaction promotes the binding of monocyte VLA-4 to vascular cell adhesion molecule-1 (VCAM-1) during the sequential cascade that regulates monocyte trafficking to inflammatory and atherosclerotic lesion[273]. In addition, platelet-derived pro-inflammatory mediators deposited on the endothelium causes increased monocyte arrest, mediated by integrin activation[38, 60, 81]. Interaction of monocytes with platelets increases the adhesive properties and recruitment of monocytes towards inflamed or atherosclerotic endothelium[111, 274, 275], which could explain our observations that more JAM-A$^{-/-}$ platelet-monocyte complexes in blood were paralleled with enhanced monocytes adhesion to the endothelium *in vivo* experiment. Platelet-activated monocytes are shifted to a pro-inflammatory phenotype, are recruited and transmigrate more efficiently at sites of inflammation, and tend to differentiate towards a dendritic cell type[265]. The interaction of platelets and pro-inflammatory macrophages has also been demonstrated to enhance macrophage activation[276]. Thus, crosstalk in monocyte–platelet complexes promotes monocyte phenotypic changes as well as extravasation, eventually promoting foam cell formation[277], which might be responsible for the increased number of Mac2^{+} macrophages in plaques of trJAM-A$^{-/-}$apoe$^{-/-}$ mice (Figure 17A).

5.4 Platelet–JAM-A deficiency promotes vascular remodeling

5.4.1 Atherosclerosis is accelerated due to platelet–JAM-A deficiency

Platelets can also initiate and propagate vascular inflammation, which may lead to the development of atherosclerotic plaques[232, 278]. In the present study, we found that JAM-A deficient platelets markedly exacerbated atherosclerosis in aortas of trJAM-A$^{-/-}$apoe$^{-/-}$ mice, compared to trJAM-A$^{+/+}$apoe$^{-/-}$ mice, fed time-course HFD (2 weeks, 6 weeks and 12 weeks), indicating that platelet-specific JAM-A-deficiency increased atherosclerotic burden in mice vasculature. Interestingly, plaque sizes in aortic roots of trJAM-A$^{-/-}$apoe$^{-/-}$ mice fed 2 weeks HFD showed significant increase, compared to trJAM-A$^{-/-}$apoe$^{-/-}$ mice, but not after 6 weeks and 12 weeks HFD (Figure 15, 16). These data indicate that the effects of JAM-A$^{-/-}$ platelets might decline during the progress of atherosclerosis in aortic root. The detailed roles of platelets in different stages of plaque formation are just emerging. Previous studies have implicated the role of platelets and their secretion products particularly in early phases of plaque development[9, 185, 236]. In early stages, the number of adherent platelets to endothelium consistently increase in the progress of atherosclerosis, which precede the development of manifest atherosclerotic lesions, as platelets might initiate inflammatory responses of endothelium[185, 232] and then recruit leukocytes to atherosclerotic predilection sites, such as the bifurcation of the carotid artery, where plaques develop[9]. Adhesion of activated platelets via P-selectin, GPIbα, and αIIbβ3 to endothelium induces endothelial expression of adhesion molecules, such as VCAM-1, E-selectin, chemokines (eg, CCL2, CXCL4 and CCL5), and matrix metalloproteinases (MMP-1, -2, and -9) [9, 60, 81, 98, 227, 231, 233]. These factors triggered by activated platelets facilitate leukocyte infiltration into the vessel wall, thus initiating and accelerating atherosclerotic plaque formation. However, we found that the numbers of Mac2^{+} macrophages and CD3^{+} T cells were significantly increased in plaques of JAM-A$^{-/-}$apoe$^{-/-}$ mice, paralleled with augmented SMCs accumulation, only at early-stage lesion (Figure 17). There was no significant difference of these inflammatory cell numbers in advanced plaques between trJAM-A$^{-/-}$apoe$^{-/-}$ mice and trJAM-A$^{+/+}$apoe$^{-/-}$ mice (Figure 18), implying that JAM-A–deficient platelet-related effects on vascular inflammation

might become secondary. Other cell types, not affected by our model of platelet-specific deletion, might gain prominence as atherosclerosis progression.

Yet the crucial roles of platelets in advanced stages of atherosclerosis were also demonstrated by previous studies. For example, inhibition of GPIb using a blocking antibody in apoe$^{-/-}$ mice dramatically reduced plaque formation after 18 weeks of HFD[9]. Additionally, repeated infusion of activated platelets exacerbated atherosclerosis[185] and integrin αIIbβ3 deficiency caused a significant reduction in plaque burden particularly after 12 weeks of HFD[221]. In this respect, although there was no significant difference of plaque formation and leukocyte infiltration in aortic roots after 12 weeks of HFD (Figure 18), there was still marked increased plasma chemokine levels (CXCL4 and CCL5) and plaque burden in vasculature of trJAM-A$^{-/-}$apoe$^{-/-}$ mice (Figure 13, 15). Thus, we hypothesize that platelet hyperreactivity, though present throughout the entire course of our study, might have the most pronounced effects on initial events of atherosclerosis, such as the induction of endothelial dysfunction, crosstalk with leukocytes in blood and propagation of leukocyte recruitment by increased plasma chemokine levels, but the effects of platelet-specific JAM-A–deficiency declined at advanced plaque formation, particularly in the aortic roots. The effects of JAM-A–deficient platelets at different stages of atherosclerotic plaque formation as well as at different locations of vasculature need to be further clarified.

Taken together, platelet–JAM-A suppresses the activity of Integrin αIIbβ3, which is functional in inflammatory platelet functions and the development of atherosclerosis, consistent with previous studies[9, 99, 221]. However, the anti- or pro-atherosclerotic roles of αIIbβ3 are still controversial as patients with genetic deficiency of αIIbβ3 not protected from atherosclerosis[224]. Our results are limited to explain the role of integrin αIIbβ3 in atherosclerotic formation directly. Atherogenic effects of blocking platelet–integrin αIIbβ3 in JAM-A$^{-/-}$apeo$^{-/-}$ mice need further investigation.

5.4.2 Platelet–JAM-A inhibits neointimal formation at early stage

The high incidence rate of restenosis is around 25-40% after PTA and stenting treatment in patients with obstructive atherosclerosis[13, 15], which is caused by neointimal formation. The neointimal lesions consist mainly of SMCs and inflammatory leukocytes[279]. Vascular injury causes endothelial denudation and exposure of extracellular matrix. Following acute vascular injury, pathophysiological processes occur rapidly, which include platelet adhesion, thrombus formation, leukocytes infiltration[16], as well as SMCs proliferation, migration and secretion of extracellular matrix[17]. Platelets, as the first adherent cells on the injured vessel wall, play a pivotal role in initiating the repair process and driving neointimal formation[38]. We found that JAM-A deficiency increased platelet adhesion on endothelium-denudated vessel wall, which might promote lesion formation at early stage of the arterial repair progress. Lots of mechanisms are involved, such as enhanced interactions of JAM-A–deficient platelets with leukocytes and injured vessel wall, increased release of chemokines and augmented recruitment of leukocytes. Furthermore, it has been found that platelet–JAM-A mediates recruitment of progenitor cells to injured vessel wall, which promotes re-endothelialization[164]. Nevertheless, the mechanisms responsible for JAM-A$^{-/-}$apoe$^{-/-}$ platelet–mediated neointimal formation have not been sufficiently elucidated.

5.4.2.1 JAM-A deficiency increases platelets adhesion on the injured vessel wall

After endothelium denuded, the extracellular matrix is exposed to the blood flow, which leads to platelet adhesion. We used HPLSM to image platelet adhesion on injured carotid artery *ex vivo* and calculated adherent platelet volume on vessel wall using the software *Lecia Application Suite X*. The data shows that the adhesion of JAM-A–deficient platelets to injured vessel wall after operation 1 hour was significantly increased, compared to control group (Figure 19).

Platelet adhesion to the injured arterial wall is mediated by several cellular adhesion molecules with sub-endothelial extracellular matrix containing adhesive molecules, such as collagen, vWF and fibrinogen[280]. Under high sheer condition of blood flow, platelets adhesion is initiated by platelet vWF receptor GPIb/V/IX and collagen receptor integrin α2β1 as well as GPVI[194, 281]. Other adhesive proteins in extracellular matrix, such as

fibronectin, thrombospondin, laminin and vitronectin, also mediate platelets interaction with the endothelial denudated vessel wall. The interaction of these adhesion molecules results in the formation of platelet layer covering on the injured vessel wall. Especially, the signal transduction of GPVI activates FcRγ chain, which in turn triggers inside-out signaling to activate integrin αIIbβ3 or α2β1 for platelet firm adhesion[195, 282, 283]. We have demonstrated that JAM-A deficiency decreased the thresholds of integrin αIIbβ3 activation, which might contribute to the enhanced integrin αIIbβ3–fibrinogen interaction. Thus, platelet JAM-A might suppress platelet adhesion to injured arterial wall via regulating αIIbβ3 activation. Subsequently, adherent platelets get activation and recruit more platelets, which cause platelet shape change and degranulation of fibrinogen, ADP/ATP as well as and thromboxane A2 (TxA2). These mediators as positive-feedback of platelet activation amplify the initial signals to result in integrin αIIbβ3-mediated aggregation of platelets. In the present *ex vivo* experiment, JAM-A as a negative regulator of integrin αIIbβ3 attenuates platelet aggregation on the endothelium– denudated vessel wall (Figure 19).

5.4.2.2 trJAM-A deficiency exacerbate neointimal formation at early stage

The functions of JAM-A in vascular remodeling have been characterized. For example, somatic deficiency of JAM-A in mice dramatically suppressed neointimal formation and leukocytes infiltration into the lesion[39]. In addition, the cell–specific roles of JAM-A in atherosclerosis have been demonstrated recently[40]. Furthermore, JAM-A deficient caused platelet hyperreactivity and promoted atherosclerosis, especially at early stage. Upon our previous studies, the same phenomenon was observed in neointimal formation. We found that platelet JAM-A deficiency increased neotintimal formation in carotid artery after wire-injury 2 weeks, but this effect was diminished after 4 weeks (Figure 20, 19). These data suggest that platelet hyperreactivtiy due to JAM-A absence initiates and propagates vascular remodeling, obviously at early stage. In later stages, these platelet-related activities might become secondary and other cell types might gain prominence. Similar with neutrophil, it was found to preferentially invade early-stage lesions, whereas the influence declines at advanced phases of vascular remodeling[236]. Acute vascular injury cause repair progress and inflammatory response, which is characterized by leukocytes

infiltration into the injured wall and SMCs migration as well as proliferation. In the present study, we found that platelet–JAM-A deficiency significantly increased the number of $CD3^+$ T cells in advanced neointimal lesion, but the data of macrophages did not show marked difference between trJAM-$A^{+/+}$apoe$^{-/-}$ and trJAM-$A^{-/-}$apoe$^{-/-}$ mice (Figure 22). These data indicate that JAM-A–deficient platelet mildly augmented inflammatory responses at advanced stage of vascular repair after acute injury, but the mechanism of platelet–JAM-A regulating vascular repair in early stages remains to be investigated.

Previous studies have shown that the mechanisms of activated platelets aggregating vascular inflammation and facilitate vascular remodeling[60]. In our mice model, we found the hyperreactivity of JAM-A–deficient platelets promoted atherosclerosis and neointimal formation, which is limited at advanced stage. In the process of vascular repair, the recruitment of leukocytes drives pathogenesis of neointimal formation[284]. Nevertheless, Platelets via leukocyte adhesion receptors or directly bridging between leukocytes and the vessel wall play critical roles for leukocyte infiltration[285, 286]. Thus, increased interactions of JAM-A–deficient platelets with leukocytes in blood flow (Figure 14) as well as endothelium-denudated vessel wall (Figure 19) would improve acute inflammatory responses. Furthermore, JAM-A deficiency increased platelet degranulation of chemoattractants, such as the elevated plasma level of CCL5 and CXCL4 (Figure 13). Deposition of chemokines from platelets to their cognate receptors on vascular cell surfaces links platelet activation to the recruitment of immune cells and exacerbates vascular diseases[88, 227, 238]. These chemokines are also localized on the vessel wall, further enhancing inflammatory responses[37, 38]. These mechanisms might explain the increased neointimal formation in trJAM-$A^{-/-}$apoe$^{-/-}$ mice at ealy stage. At later stages, re-endothelialization might diminish platelet adhesion on vessel wall, thus suppressing the effects of JAM-A–deficient platelets on neointimal formation. However, the role of platelet–JAM-A in neointimal formation after arterial injury remains to be elucidated.

6 Summary

Platelets play an essential role in hemostasis and also act as immune cells regulating inflammation. The deficiency of platelet function suppresses atherosclerosis in hyperlipidemic mice[60, 77]. In the present study, platelet hyperreactivity due to JAM-A absence promoted vascular remodeling, particularly in early phases.

Although plenty of mechanisms are responsible for platelet activation, little is known about the signaling pathways that negatively regulate platelet function. Recently, JAM-A was found to inhibit platelet outside-in signaling through integrin αIIbβ3. Consistent with previous studies (*Naik et al., Blood 2013*), we also report that JAM-A suppresses platelet reactivity through down-regulating c-Src activity in integrin αIIbβ3 outside-in signaling.

Specific deletion of JAM-A in platelets led to an enhanced response to various agonists, such as ADP or thrombin. As a result, interactions of JAM-A–deficient platelets with leukocytes as well as with inflamed vascular endothelium are increased. Remarkably, tirofiban as an inhibitor of integrin αIIbβ3 normalized the hyperreactivity of JAM-A–deficient platelets. Furthermore, increased plasma levels of chemokines (CCL5 and CXCL4) and an increased recruitment of leukocytes was found in propagated plaques of trJAM-A$^{-/-}$apoe$^{-/-}$ mice. Taken together, deletion of JAM-A caused a gain-of-function in platelets with lower activation thresholds. This promoted vascular inflammation and increased plaque formation, particularly in early stages.

JAM-A–deficient platelets gained an increased aggregation on endothelium-denudated vessel wall, which might accelerate neoinitmal formation at early stage after vascular injury, but not at advanced stages. The detailed mechanisms of JAM-A–deficient platelets facilitating neointimal formation remain to be investigated.

Taken together, Hyperreactivity of JAM-A–deficient platelets aggravates vascular inflammation and remodeling in hyperlipidemic mice, particularly at early stages. This highlights the detrimental role of activated platelets in preclinical phases of cardiovascular disease.

7 References

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8 Contribution statement

Sections 4.1.1, 4.1.2 JAM-deletion on platelets; JAM-deletion in arterial wall

The experiments in this chapter were designed, performed and analyzed by Dr. Ela Karshovska and Mr. Zhen Zhao.

Section 4.1.3 Related adhesion molecules expression on platelets

The experiments in this chapter were designed and performed by Dr. Ela Karshovska and Mr. Zhen Zhao and the data were collected and analyzed by Dr. E. Karshovska and Rory R. Koenen.

Section 4.2 Platelets and white blood cells in mouse blood

The measurements in this chapter were performed by Dr. Ela Karshovska and Mr. Zhen Zhao and the data were collected and analyzed by Dr. E. Karshovska.

Section 4.3 Lipid metabolism after trJAM-A deletion

The measurements in this chapter were designed and performed by Drs. Xavier Blanchet, Ela Karshovska and Mr. Zhen Zhao and the data were collected and analyzed by Dr. E. Karshovska.

Sections 4.4.1, 4.4.2, 4.4.3 JAM-A-deficiency increases platelet aggregation; JAM-A negatively regulates platelet activity through integrin αIIbβ3; JAM-A inhibits c-Src activity in integrin αIIbβ3 outside-in signaling

The measurements in this chapter were designed and performed by Dr. Ela Karshovska and Mr. Zhen Zhao and the data were collected and analyzed by Dr. E. Karshovska and Rory R. Koenen.

Section 4.4.4 PTP1 catalyzes JAM-A de-phosphorylation after platelet activation

The experiments in this chapter were designed and performed by Dr. Xavier Blanchet, Rory R. Koenen, Mr. Zhen Zhao and Dr. Ela Karshovska. Data were collected and analyzed by Dr. Rory R. Koenen and Ela Karshovska.

Section 4.5.1 Augmented adhesion of JAM-A-deficient platelets to atherogenic vessel wall

The experiments in this chapter were designed and performed by Dr. Martin Schmitt, Ela Karshovska and Mr. Zhen Zhao. Data were collected and analyzed by Dr. Ela Karshovska.

Section 4.5.2 JAM-A-deficient platelets increase plasma levels of chemokines

The experiments in this chapter were performed by Dr. Ela Karshovska, Xavier Blanchet and Mr. Zhen Zhao. Data were collected and analyzed by Dr. Ela Karshovska.

Sections 4.5.3, 4.6.1, 4.6.2 JAM-A deficiency enhances interaction of platelets with leukocytes; JAM-A-deficient platelets propagate aortic atherosclerosis; Platelet JAM-A deficiency enhances early-stage atherosclerosis in aortic root

The experiments in this chapter were designed and performed by Dr. Ela Karshovska and Mr. Zhen Zhao. Data were collected and analyzed by Dr. Ela Karshovska.

Sections 4.6.3, 4.6.4 trJAM-A absence promotes leukocyte infiltration into early-stage plaques; Platelet JAM-A deficiency did not alter cell composition in advanced plaques

The experiments in this chapter were designed and performed by Dr. Ela Karshovska and Mr. Zhen Zhao. Data were collected and analyzed by Dr. Ela Karshovska and Mr. Zhen Zhao.

Section 4.7.1 JAM-A deficiency encourages platelet adhesion on injured arterial wall

The experiments in this chapter were designed and performed by Mr. Zhen Zhao and Dr. Remco Megens. Data were collected and analyzed by Mr. Zhen Zhao.

Section 4.7.2 Platelet JAM-A deficiency increases neontimal formation at early stage

The experiments in this chapter were designed and performed by Mr. Zhen Zhao and Dr. Ela Karshovska. Data were collected and analyzed by Mr. Zhen Zhao.

Section 4.7.3 Platelet JAM-A does not affect neointimal formation at advanced stage

The experiments in this chapter were designed and performed by Mr. Zhen Zhao and Dr. Ela Karshovska. Data were collected and analyzed by Mr. Zhen Zhao.

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